Article:

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Supporting Information

Discovery of a Fungal Copper Radical Oxidase with High Catalytic Efficiency Towards 5-hydroxymethylfurfural and Benzyl Alcohols for Bioprocessing.

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Supporting Tables

Table S1: Initial activity screens* of *Cgr*AAO-WT and its variants

	Substrate	Specific activity (µmole.min^{-1}.mg^{-1})		
		*Cgr*AAO-WT	CgrAAO-Y334F	*Cgr*AAO-Y334W
Carbohydrates	D-Galactose (300 mM)	1.80 ± 0.03	4.1 ± 0.1	34.9 ± 0.3
	D-Lactose (300 mM)	1.93 ± 0.05	4.4 ± 0.1	12.6 ± 0.3
	Melibiose (300 mM)	9.9 ± 0.7	20.6 ± 1.2	46.9 ± 0.5
	Raffinose (300 mM)	8.5 ± 0.1	38.3 ± 1.1	51.4 ± 0.3
	D-Glucose (300 mM)	0.050 ± 0.002	n.m.§	n.m.§§
	D-Xylose (300 mM)	0.950 ± 0.005	3.80 ± 0.08	0.88 ± 0.01
	L-Arabinose (300 mM)	0.74 ± 0.01	n.m.§	n.m.§
	D-Ribose (300 mM)	0.37 ± 0.01	0.67 ± 0.01	0.38 ± 0.03
	D-Fructose (300 mM)	0.117 ± 0.006	n.m.§	n.m.§
	D-Mannose	0.066 ± 0.004	n.m.§	0.070 ± 0.006
	Sucrose (300 mM)	0.045 ± 0.001	n.m.§	n.m.§
	Maltose (300 mM)	0.051 ± 0.002	n.m.§	n.m.§
	Cellobiose (300 mM)	0.115 ± 0.002	n.m.§	0.14 ± 0.01
	Carob Galactomannan (2.5 mg.mL^{-1})	0.43 ± 0.02	0.90 ± 0.02	0.063 ± 0.004
	Xyloglucan (2.5 mg.mL-1)	0.060 ± 0.002	n.m.§	n.m.§
Polyols	Glycerol (300 mM)	7.2 ± 0.4	18.4 ± 0.3	12.3 ± 0.4
	Sorbitol (300 mM)	0.760 ± 0.007	0.60 ± 0.03	0.32 ± 0.02
Diols	1,2-Propanediol (300 mM)	4.02 ± 0.04	6.3 ± 0.2	0.35 ± 0.02
	1,3-Propanediol (300 mM)	10.9 ± 0.1	42.7 ± 0.9	1.87 ± 0.03
	1,4-Butanediol (300 mM)	2.6 ± 0.1	n.m.§	2.2 ± 0.1
Aldehyde	Methyl glyoxal (5 mM)	n.m.§	1.3 ± 0.1	n.m.§
Primary Alcohols	Methanol (300 mM)	0.81 ± 0.02	1.50 ± 0.07	0.19 ± 0.01
	Ethanol (300 mM)	0.42 ± 0.04	0.70 ± 0.01	0.050 ± 0.002
	1-Butanol (300 mM)	0.85 ± 0.02	n.m.§	n.m.§
	1-Propanol (300 mM)	0.50 ± 0.01	n.m.§	n.m.§
Secondary Alcohols	2-Propanol (10 mM)	0.036 ± 0.002	n.m.§	n.m.§
	1-Phenyl Ethanol (10 mM)	n.m.§	n.m.§	n.m.§
	2-Phenyl Ethanol (10 mM)	n.m.§	n.m.§	n.m.§
Benzyl Alcohols	Benzyl alcohol (5 mM)	3.4 ± 0.1	10.6 ± 0.1	1.24 ± 0.06
	m-Anisyl alcohol (5 mM)	3.1 ± 0.2	8.2 ± 0.2	1.63 ± 0.05
	p-Anisyl alcohol (5 mM)	2.9 ± 0.1	6.6 ± 0.2	1.03 ± 0.02
	Veratryl alcohol (5 mM)	3.71 ± 0.05	10.8 ± 0.3	1.48 ± 0.08
	Cinnamyl alcohol (5 mM)	2.8 ± 0.2	6.4 ± 0.3	0.64 ± 0.08
	4-Hydroxy benzyl alcohol (5 mM)	3.3 ± 0.1	7.8 ± 0.4	1.3 ± 0.1
	Coniferyl alcohol (5 mM)	n.m.§	n.m.§	n.m.§
Furans	HMF (5 mM)	26.4 ± 1.1	16.4 ± 0.9	1.39 ± 0.06
	HMFCA (5 mM)	2.8 ± 0.3	2.1 ± 0.1	0.66 ± 0.04
	DFF (5 mM)	0.0010 ± 0.0001	0.051 ± 0.001	0.100 ± 0.005
	FFCA (5 mM)	0.0020 ± 0.0001	0.003 ± 0.001	0.0030 ± 0.0001

* Measurements were performed in triplicates at 25 °C in 100 mM sodium phosphate buffer pH 7 using the HRP/ABTS assay. Activities were monitored using concentrations indicated within parentheses for each substrate.

§No activity detected with a specific activity limit of detection of 9 x 10^{-4} µmole.min^{-1}.mg^{-1} using 65 µmole of protein, which is 5-fold

Table S2: EPR spin Hamiltonian parameters from simulations of cw X band spectra for *Cgr*AAO-WT, -Y334F and –Y334W[a]

		*Cgr*AAO-WT	*Cgr*AAO-Y334F	*Cgr*AAO-Y334W
***g* values**	g_1	2.059	2.059	2.049
	g_2	2.072	2.072	2.061
	g_3	2.278	2.278	2.275
A_{Cu} (MHz)	$\lvert A_1\rvert$	40	40	50
	$\lvert A_2\rvert$	45	40	50
	$\lvert A_3\rvert$	530	530	515
SHF A_N principal values (MHz) *		43, 43 ±3	43, 43 ±3	45, 45 ±3
A_{cu} strains (MHz)		55, 65, 130	35, 75, 130	50, 65, 130
Line widths (mT)		0.7, 0.7	0.7, 0.7	0.8, 0.8
Frequency (GHz)		9.2986	9.2995	9.2982

* error estimated from quality of simulated fits

[a]. Spectra were recorded in the presence of 10% glycerol in 100 mM Na phosphate buffer pH 7.0. For coupled nitrogen nuclei, only the principal coupling value could be determined from the simulations of the superhyperfine (SHF); the two values refer to the two different N nuclei.

Table S3: Comparison of catalytic parameters of *Cgr*AAO with other enzymes acting on HMF and its derivatives*

	HMF			DFF			HMFCA			FFCA		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
Bacterial HMFO[a]	1.4	9.9	7.1 x 10^3	1.7	1.6	940	73	8.5	120	NM	NM	<10
***Per*AAO**[b]	1.6 ± 0.2	0.33 ± 0.01	220 ± 43	3.3 ± 0.2	0.52 ± 0.01	158.0 ± 9.2	NM	NM	NM	NM	NM	NM
MtGLOx[c]	20.2 ± 9.0	15.9	982	NM	NM	NM	NA	NA	NA	NA	NA	NA
Pciglox1[d]	15.66 ± 2.35	1.59 ± 0.12	101.66 ± 0.01	4.38 ± 0.1	0.54 ± 0.24	124.39 ± 0.01	NA	NA	NA	0.85 ± 0.14	0.03 ± 0.01	38.55 ± 0.01
Pciglox2[d]	5.87 ± 2.04	0.56 ± 0.09	96.04 ± 0.01	0.21 ± 0.04	4.80 ± 0.24	2.34 ± 0.01 x 10^4	NA	NA	NA	1.40 ± 0.39	2.02 ± 0.03	1.40 ± 0.01 x 10^3
Pciglox3[d]	6.35 ± 1.32	0.75 ± 0.07	118.35 ± 0.01	0.18 ± 0.05	1.28 ± 0.09	7.30 ± 0.01 x 10^3	NA	NA	NA	0.61 ± 0.58	0.04 ± 0.01	72.03 ± 0.01
***Cgr*AAO**[e]	6.5 ± 0.3	126.0 ± 1.5	1.94 ± 0.09 x 10^4	NM	NM	NM	26.9 ± 3.0	28.3 ± 1.3	1.1 ± 0.1 x 10^3	NM	NM	NM

* NM not measurable; NA non assessed

[a] Kinetic data from [1]; [b] Kinetic data from [2]; [c] Kinetic data from [3]; [d] Kinetic data from [4]; [e] Kinetic data derive from Table 1

Table S4 : PCR primers[a]

	Primers name	Primers sequence 5' - 3'
Mutagenesis	*Cgr*AAO-Y334W-f	GGTGGGCTTggTCAGGTGAGC
	*Cgr*AAO-Y334W-r	AATAGTGAAGACCTTACCATTAC
	*Cgr*AAO-Y334F-f	GGTGGGGCTTtTTCAGGTGAG
	*Cgr*AAO-Y334F-r	AATAGTGAAGACCTTACCATTAC

[a]. Primer sequences used for site directed mutagenesis. Mutated bases are in lowercase.

Supporting Figures

A

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FgrGalOx|Q01745        GLGRWGPTIDLPIVPAAAAIEP---TSGRVLMWSSYRNDAFGGSPGGI-TLTSSWDPSTG   56
CgrAlcOx|EFQ30446      NVGKWGPMVKFPVVPVAVALVP---ETGNLLVWSSGWPNRWTTAGNGK-TYTSLYNVNTG   56
CglAlcOx|ELA25906      GLGQWSPLIKFPVVPVSVALLP---ESGNLLVWSSGWPNRWTTAGNGK-TYTSLYNVQTG   56
CgrRafOx|EFQ36699      QNGQWSPIQTLPLNPVAAYLVPAYPVVQDFLSFSSFSPFTFGGGPAYFNTAFMRYNIKSS   60
PorAlcOx|XP_003719369  SAGQWGPIVKFPVVPVSVALIP---ESGDLIVWSSGWPDRFTNGGNGK-TYTSIYNVQTG   56
ChiAlcOx|OBR05259      NVGQWGPMVKFPVVPVAVALLP---ETGNMLVWSSGWPNRWTTAGNGK-TYTSIYDVKTG   56
PruAA5_2A|CAP96757     NGGVWGPTIDLPVVAVSGAVIP---ETNEVLVWSSWAKDDYLHS-RGY-TLTAVWNMNDN   55
CgrAAO|EFQ27661        VKGKWGDLIRLPVIPVAAYIVPSYPEPSRLLFFSSWSNDAFSGA-SGM-TQFGDYDFATG   58

FgrGalOx|Q01745        IVSDRTVTVTKHDMFCPGISMDGNGQIVVTGGNDAKKTSLYDSS---SDSWIPGPDMQVA  113
CgrAlcOx|EFQ30446      NISDAIVQNTQHDMFCPGTSLDADGRIIVTGGSSAAKTSVLDFKKGESSPWTPLSNMQIS  116
CglAlcOx|ELA25906      NVSDAVIQNTQHDMFCPGTSLDAEGRIIVTGGSSAAKTSVLDFKNGESSSWTALSNMQIS  116
CgrRafOx|EFQ36699      AASQFNVAETKHDMFCPGMNHLADGRLVINGGNTDAAVTIYDPF---ANTWTRAANMNMG  117
PorAlcOx|XP_003719369  NVSEAIIQNTSHDMFCPGTSMDEFGRIVVTGGSGAAKTSVFDFQNGQRSPWMPASDLTNP  116
ChiAlcOx|OBR05259      KVSDALIQNTQHDMFCPGTSMDENGRIIVTGGSSASKTSVLDFKKGEFSSWTPLSNMQIS  116
PruAA5_2A|CAP96757     SVTQRKVQETHHDMFCSGMSYDGKGELLVTGGNNDKSTSIFDPA---SGKWTEGNTMIIT  112
CgrAAO|EFQ27661        AISQRTVTNTHHDMFCPGISQLEDGRILIQGGSDADTVSIYDPA---TNEFTRGPNMTLA  115

FgrGalOx|Q01745        RGYQSSATMSDGRVFTIGGSWSGGV------FEKNGEVYSPSSKTWTSLPNAKVNPMLTA  167
CgrAlcOx|EFQ30446      RGYQSSCTTSEGKIFVIGGSFSGAG-------TRNGEVYDPKANTWTKLAGCPVKPLVMQ  169
CglAlcOx|ELA25906      RGYQSSCTTSEGKIFVIGGSFSGAG-------TRNGEIYDTATNKWTKLAGCPVKPLVMQ  169
CgrRafOx|EFQ36699      RGYQSSVTLSDGRGFTIGGSYTGGIGGQNGTPMKNGEVYDPKLNKWTALPGALVAPMLTT  177
PorAlcOx|XP_003719369  RGYQSSVTTSEGKIFTIGGTFSGNG-------KRDGEVYDVNANKWTKLPGCPATIMRVA  169
ChiAlcOx|OBR05259      RGYQSSCTTSEGKIFVIGGSFSGAG-------RRDGEVYDPKANTWTKLAGCPVKPLVMQ  169
PruAA5_2A|CAP96757     RGYQASATIADGRVFIIGGSWNGGTN------YDKDGEIYDPDTEKYSFLKNALVRPMWTD  167
CgrAAO|EFQ27661        RGYQTSCTLSNGKVFTIGGAYSGER------VGKNGEVYDPVANAWTYLPGADFRPMLTN  169

FgrGalOx|Q01745        -DKQGLYRSDNHAWLFGWKKGSVFQAGPSTAMNWYYTSGSGDVKSAGKRQSNRGVAPDAM  226
CgrAlcOx|EFQ30446      ----RGMFPDSHAWLWSWKNGSVLQAGPSKKMNWYDTKGTGSNTPAGLRG----TDEDSM  221
CglAlcOx|ELA25906      ----LGMFPDSHAWLWSWKNGSVLQAGPAKQMNWYDTKGTGANTPAGLRG----ADQDSM  221
CgrRafOx|EFQ36699      YDNAGAWRTDNHAWLYAWSNGSVFQAGPSKNMNWYSTSGQGSVKGAGQRN----TQNDQM  233
PorAlcOx|XP_003719369  ----GGLYPDSHTWLWGWKDGFVLQAGPSKKMNWFDTKGTGGNKPAGTRG----ADQDSM  221
ChiAlcOx|OBR05259      ----RGLFPDSHAWLWSWKNGTVLHAGPAKQMNWYYTKGTGANTPAGLRG----ADDDSM  221
PruAA5_2A|CAP96757     -DQDSGYRRDSHGWLFGWKNDTVFQGGPSKNMNWYYTHGDGDQKPAGTRA----DANDSM  222
CgrAAO|EFQ27661        -DHEGIWREDNHAWLFGWKNGSIFQAGPSKDQHWYGIQGNGTVAKAATR-----DDDDAM  223

FgrGalOx|Q01745        CGNAVMYDAVKGKILTFGGSPDYQDSDATTNAHIITLG-EPGTSPNTVFA----SNGLYF  281
CgrAlcOx|EFQ30446      CGVSVMYDAVAGKIFTYGGGKGYTGYDSTSNAHILTLG-EPGQAVQVQK-----LANGKY  275
CglAlcOx|ELA25906      CGVSVMYDAVAGKIFTYGGGKGYTGYQSTSNAHILTLG-EPGQQVQVQK-----LQNGQY  275
CgrRafOx|EFQ36699      CGVTVMYDS--GKIFAAGGAQSYSDDKALYAAHRITLN-GVNQSPTVQQ-----LPNAKY  285
PorAlcOx|XP_003719369  CGVTAMYDAAAGKVFTYGGGLRYTGESGSNAAHVLTLPDTPGDLVAVER-----VSDGQF  276
ChiAlcOx|OBR05259      CGVSVMYDAVAGKIFTYGGGKAYTGVASSSNAHILTLG-EPGQAVQVQK-----LQNGKF  275
PruAA5_2A|CAP96757     SGNAVMFDAVNGKIITFGGSPSYENSYATTDAYLIEID-EPGSQPKVTAAKNPNGEGMAY  281
CgrAAO|EFQ27661        CGVWVMYDAVAGKIFSAGGSPDYTDSPATQRAHITTIG-EPNTPAEVER-----VADMGF  277

FgrGalOx|Q01745        ARTFHTSVVLPDGSTFITGGQRRGIPFEDSTPVFTPEIYVPEQDTFYKQNPNSIVRVYHS  341
CgrAlcOx|EFQ30446      NRGFANAVVMPDGKIWVVGGMQKMWLFSDTTPQLTPELFDPATGSFTPTTPHTVPRNYHS  335
CglAlcOx|ELA25906      NRGFANAVVMPDGKIWVVGGMKQMALFSDATPQLTPELFDPATGKFTPTAAHTVPRNYHS  335
CgrRafOx|EFQ36699      ARIFAQAIVLPNGQVFVTGGQAYAAGFTDTLSVLQAEVYDPVANTFTPVAALAVPRNYHS  345
PorAlcOx|XP_003719369  GRGYHNAVVLPDGKVFVVGGMSRMALFSDGSPQLFPEIWDPATGGFTTMRPHTIPRNYHS  336
ChiAlcOx|OBR05259      NRGFANAVVMPDGKIWVVGGMRQMQLFSDSTPQLTPELFDPATGVFTPTTPHTVPRNYHS  335
PruAA5_2A|CAP96757     ARTFHTSVVLPDGGVFTAGGQSYGVPFNDSNAHLTPELYDPKTNQFNEQQPNSIVRVYHS  341
CgrAAO|EFQ27661        PRGFANAVVLPDGQVLVTGGQRMSLVFTNTDGILVAELFNPETREWKQMAPMAVPRNYHS  337

FgrGalOx|Q01745        ISLLLPDGRVFNGGGGLCG-----------DCTTNHFDAQIFTPNYLYNSNGN-LATRPK  389
CgrAlcOx|EFQ30446      TALLMADGTIWSGGGGLCG-----------ANCKENHFDGQFWSPPYLFEADGVTPAKRPV  385
CglAlcOx|ELA25906      TALLMADGTIWSGGGGLCG-----------AGCAANKFDGQFWSPPYLFEADGKTPAKRPV  385
CgrRafOx|EFQ36699      TGLLLPDGRVMNGGGGLCYVGG------G-CNSGNHPDLQFWTPPYMFDARGN-PATRPQ  397
PorAlcOx|XP_003719369  TAMLMADGTVFSGGGGLCG-----------AGCSANHFDGQFFSPPYLFQADGRTPAQRPV  386
ChiAlcOx|OBR05259      TALLMADATIWSGGGGLCG-----------ANCKENHFDGQFWSPPYLFEADGKTPAKRPV  385
PruAA5_2A|CAP96757     ISLLLPDGRVFNGGSGLGV-----------SAPTNHFDAQIYSPHYLFNQDGS-LATRPT  389
CgrAAO|EFQ27661        VSILLPDATVFSGGGGMCWVQNVGDSTAGCDKTVDHSDGEIFEPPYLFNEDGS-RAARPV  396

FgrGalOx|Q01745        ITRTSTQS-------VKVGGRITISTD--SSISKASLIRYGTATHTVNTDQRRIPLTLTN  440
CgrAlcOx|EFQ30446      IQSLSDT-------AVRAGAPITITMQDA-GAYTFSMIRVSATTHTVNTDQRRIPLDGQD  437
CglAlcOx|ELA25906      IESLSDE-------TVKAGAALTINMQDE-GKYTFSMIRVSATTHTVNTDQRRIPLDGQD  437
CgrRafOx|EFQ36699      ISSISASQQSGNQVRVSPGGKLTVVLGSSGANLGHVLVRMGSGTHSIDTDQRRIPLTVYS  457
PorAlcOx|XP_003719369  IRSLGPASGANGAVEVRAGDQVTVTMQDA-GAYSFSMIRTGSTTHTVNTDSRRIPLAGQD  445
ChiAlcOx|OBR05259      IQDLSET-------TVKAGAAITVTMQDA-GAYTFSMIRVSATTHTVNTDQRRIPLDGQD  437
PruAA5_2A|CAP96757     IDSVANKN-------LRAGDKLSISAS--IDVKNASLIRYGTTTHTVNTDQRRISLDSWT  440
CgrAAO|EFQ27661        ISAISADP-------IKAGATLTFTVEGVEGQGTAALIRLGSVTHSVNSDQRRVPLNVTV  449

FgrGalOx|Q01745        NG-GNSYSFQVPSDSGVALPGYWMLFVMNSAGVPSVASTIRVTQ   483
CgrAlcOx|EFQ30446      GGDGKSFTVNVPNDYGVAIPGYYMLFAMNEAGVPCVAQFFKVTL   481
CglAlcOx|ELA25906      GGDGKSFSVNMPSDYGVVIPGYYMMFAMNEAGTPCVAKFFKVSL   481
CgrRafOx|EFQ36699      TN-GNTVALSIPNDNGVVPPGFWYYFAVAPSGVHSIGLTVNVLA   500
PorAlcOx|XP_003719369  VGGG-SYVVTVPSDYGIATPGYYMLFALSEAGVPAVAKFFRVGL   488
ChiAlcOx|OBR05259      GGDGQAFTVNVPADYGVAVPGYYMLFAMNEAGVPCVAKFFKVSL   481
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B)

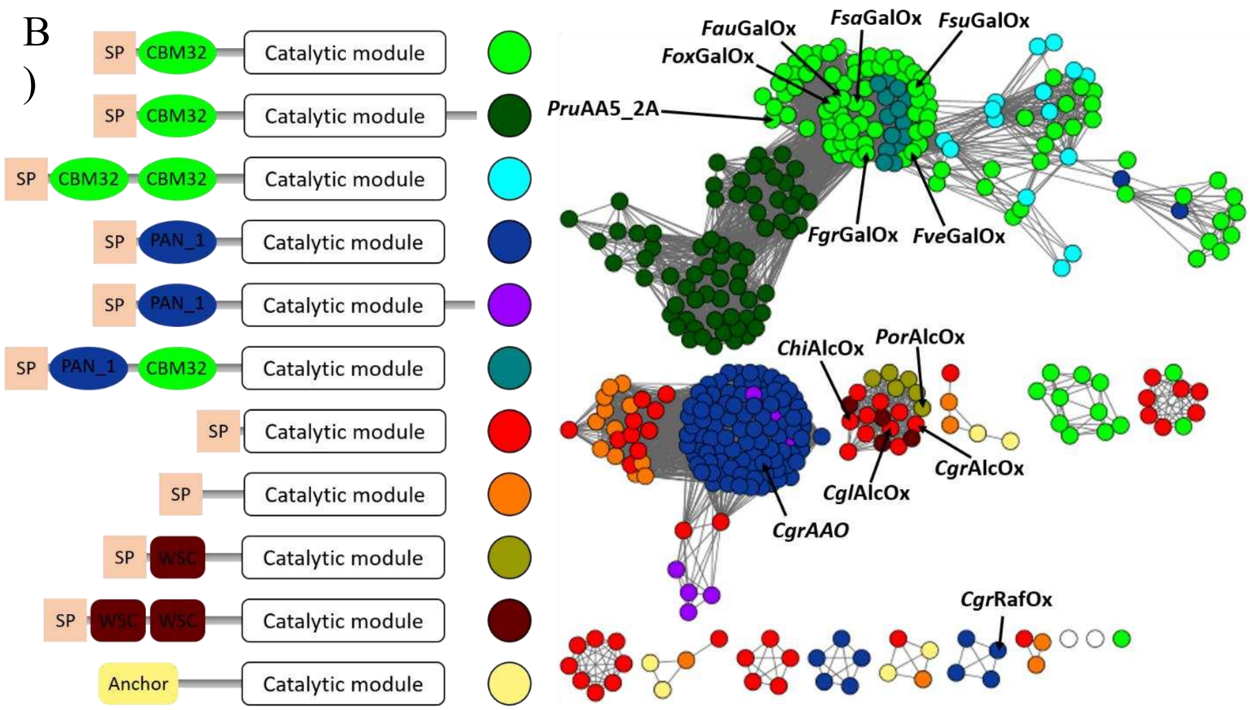
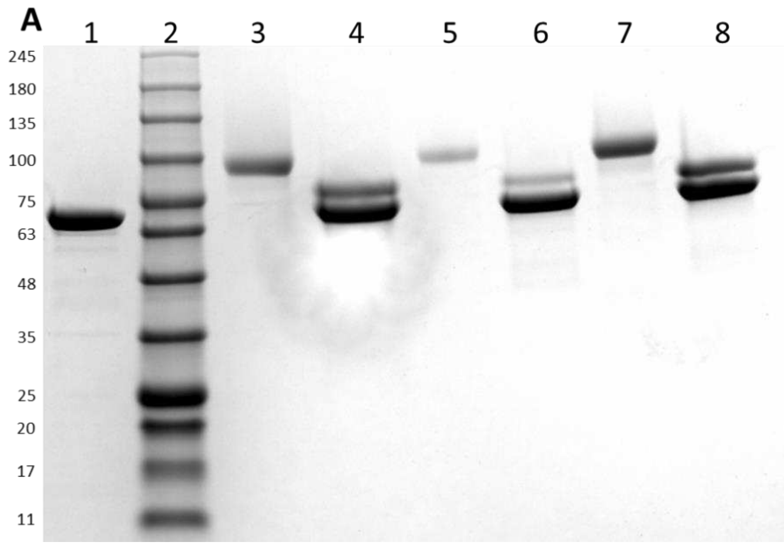


Figure S1. (A) Sequence alignment of *Colletotrichum graminicola* aryl alcohol oxidase (*Cgr*AAO) with characterized AA5_2 members. (B) Sequence similarity network at an alignment score cut-off of 10^{-550} of 392 catalytic modules from the AA5_2 subfamily with their corresponding modularity. For each panel, predicted native signal peptides and additional N-terminal modules have been removed. Conserved active-site catalytic residues and residues involved in substrate recognition are highlighted in yellow and green, respectively (A). Each node is colored according to its modularity. Catalytic modules are shown in white, carbohydrate binding modules are in green [5], PAN_1 domains are blue [6], WSC are brown [7] and GPI anchor are yellow (B). *Cgr*AlcOx = *Colletotrichum graminicola* alcohol oxidase, *Cgl*AlcOx = *Colletotrichum gloeosporioides* alcohol oxidase, *Cgr*RafOx = *Colletotrichum graminicola* raffinose oxidase, *Pru*AA5_2A = *Penicillium rubens Wisconsin 54–1255* AA5_2 oxidase, *Fgr*GalOx = *Fusarium graminearum* galactose oxidase, *Chi*AlcOx = *Colletotrichum higginsianum* alcohol oxidase and *Por*AlcOx = *Pyricularia oryzae* alcohol oxidase.



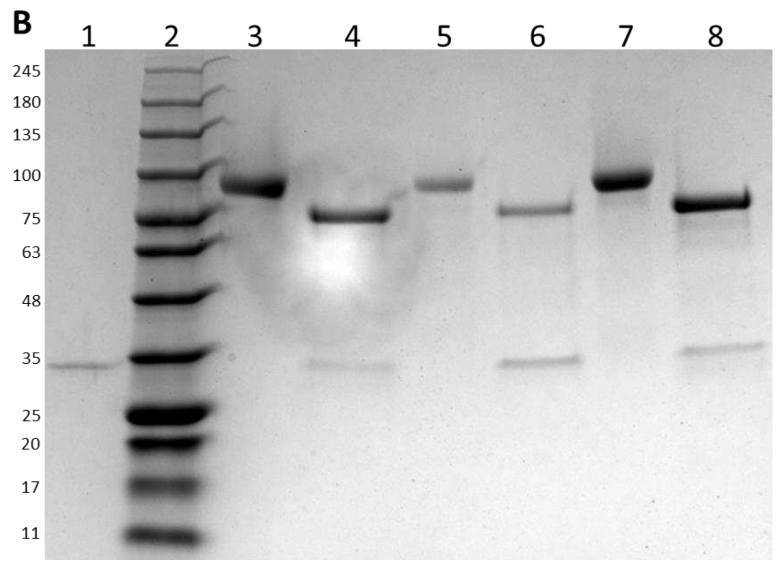


Figure S2. SDS-PAGE of *Cgr*AAO-WT, *Cgr*AAO-Y334F and *Cgr*AAO-Y334W & *N*-deglycosylation studies.

Enzymes were *N*-deglycosylated under denaturing conditions with either PNGaseF (A) or EndoH (B).

(A): 1: EndoH, 2: molecular weight marker, 3: *Cgr*AAO-WT (5 µg), 4: *Cgr*AAO-WT (5 µg) + EndoH, 5: *Cgr*AAO-Y334W (5 µg), 6: *Cgr*AAO-Y334W (5 µg) + EndoH, 7: *Cgr*AAO-Y334F (5 µg), 8: *Cgr*AAO-Y334F (5 µg) + EndoH

(B): 1: PNGaseF, 2: molecular weight marker, 3: *Cgr*AAO-WT (5 µg), 4: *Cgr*AAO-WT (5 µg) + PNGaseF, 5: *Cgr*AAO-Y334W (5 µg), 6: *Cgr*AAO-Y334W (5 µg) + PNGaseF, 7: *Cgr*AAO-Y334F (5 µg), 8: *Cgr*AAO-Y334F (5 µg) + PNGaseF

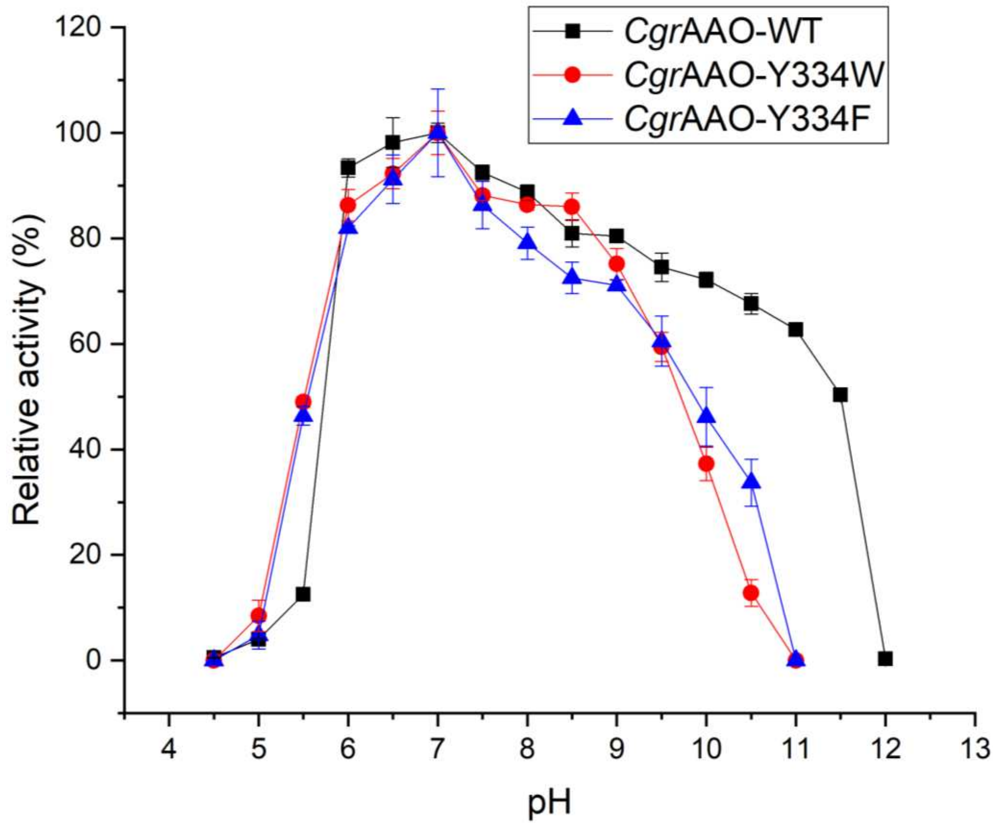


Figure S3. pH-rate profiles of *Cgr*AAO-WT and mutants. Data are represented as means ± standard deviations (n = 3). Activities were determined by the HRP/ABTS assay monitoring absorbance at 420 nm using 50 mM HMF for *Cgr*AAO-WT and *Cgr*AAO-Y334F, and 500 mM melibiose for *Cgr*AAO-Y334W. pH rate profiles were determined after 1-min incubations at the desired pH, pH range 4-6 was maintained using 100 mM phosphate-citrate buffers, pH range 6-8 was maintained using 100 mM phosphate buffers and pH range 8- 12 was maintained using glycine-sodium hydroxide buffers.

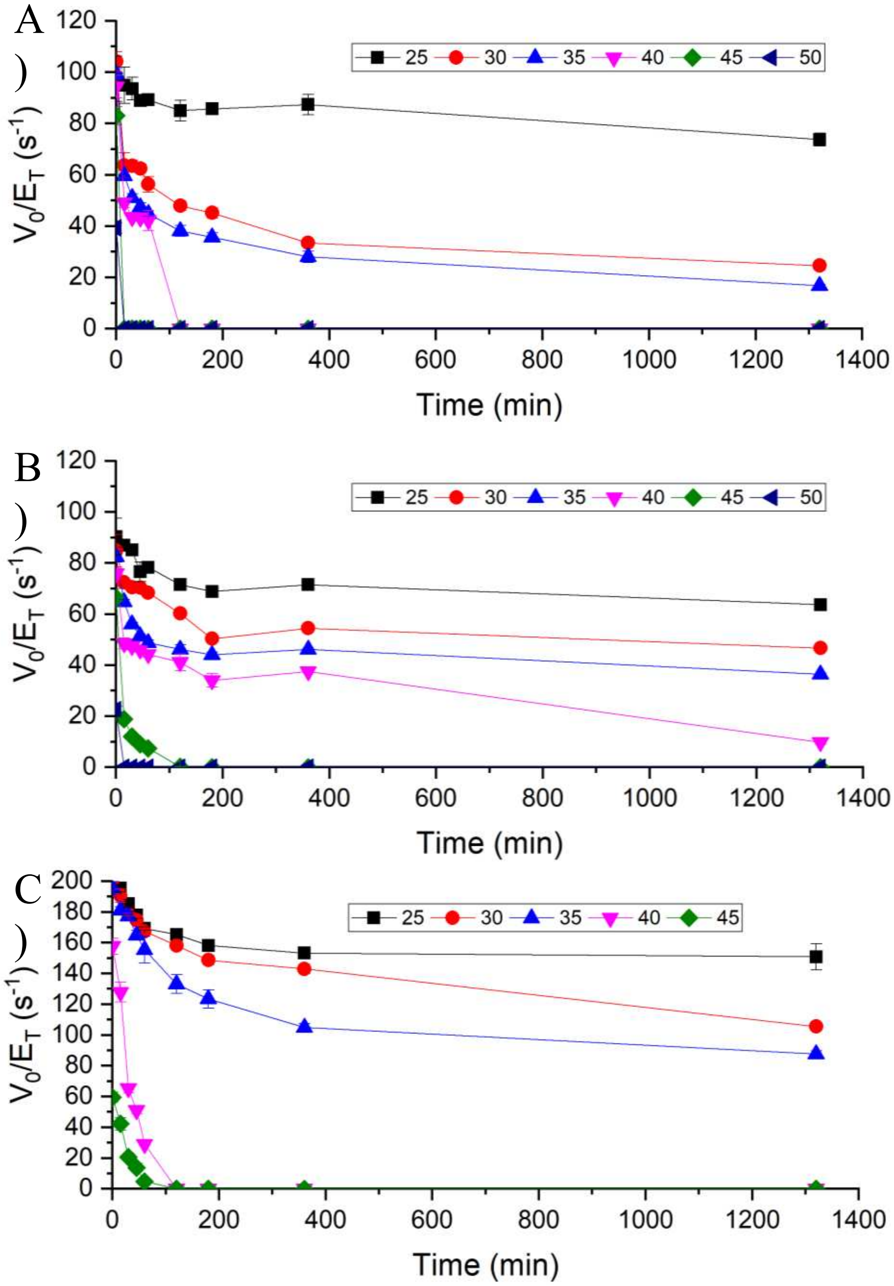
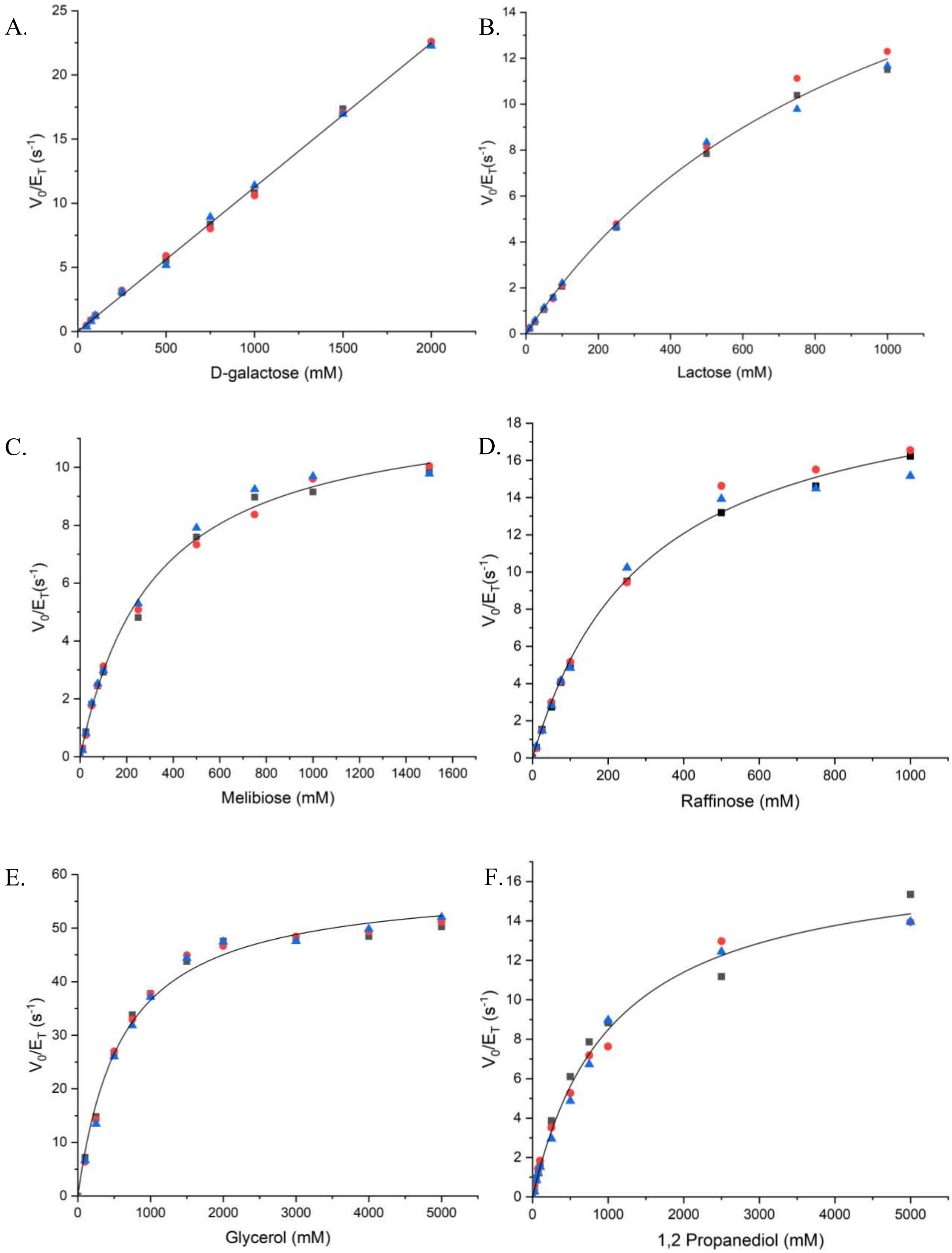
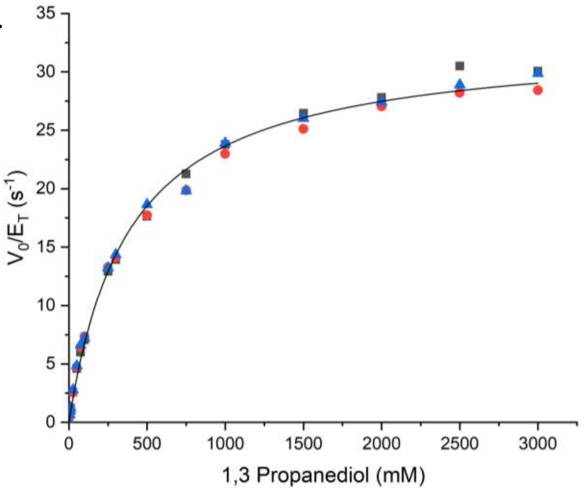


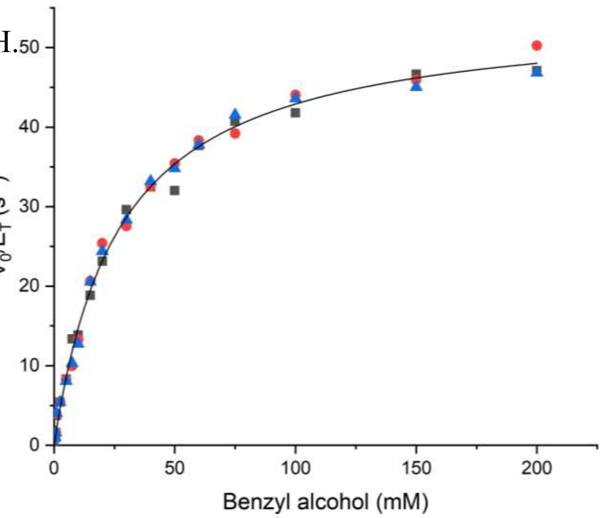
Figure S4. Temperature stability. A) *Cgr*AAO-WT; B) *Cgr*AAO-Y334W; C) *Cgr*AAO-Y334F. Data are represented as means ± standard deviations (n = 3). Activities values were determined by the coupled HRP/ABTS at each temperature, maintained by a gradient thermocycler, using 50 mM HMF for *Cgr*AAO-WT and *Cgr*AAO-Y334F, and 500 mM melibiose for *Cgr*AAO-Y334W.


A.
V0/ET (s-1)
D-galactose (mM)
B.
V0/ET(s-1)
Lactose (mM)
C.
V0/ET(s-1)
Melibiose (mM)
D.
V0/ET(s-1)
Raffinose (mM)
E.
V0/ET (s-1)
Glycerol (mM)
F.
V0/ET (s-1)
1,2 Propanediol (mM)

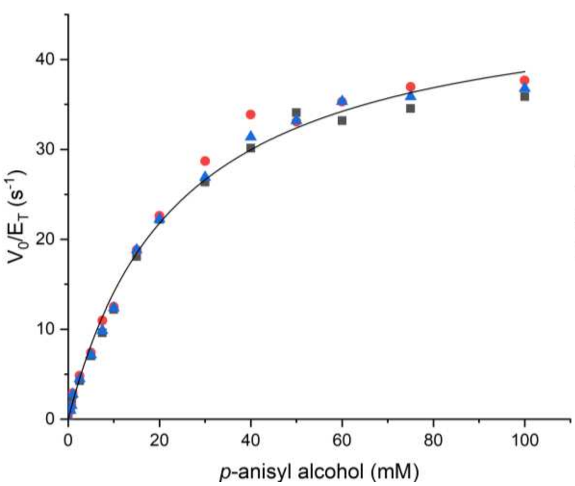
G.


V_0/E_T (s^{-1})
0 5 10 15 20 25 30 35
0 500 1000 1500 2000 2500 3000
1,3 Propanediol (mM)

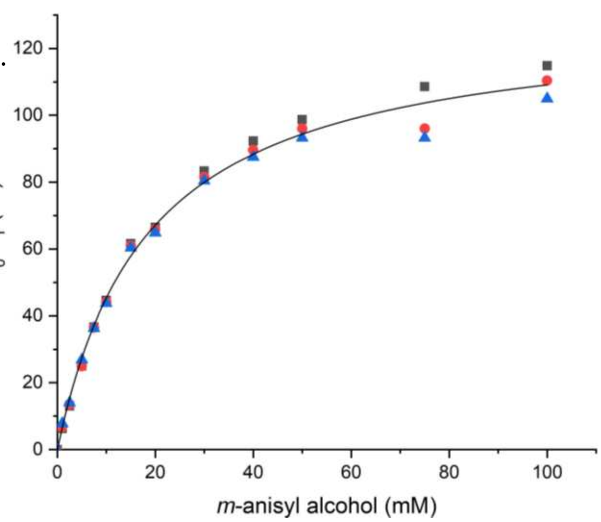
H.


V_0/E_T (s^{-1})
0 10 20 30 40 50
0 50 100 150 200
Benzyl alcohol (mM)

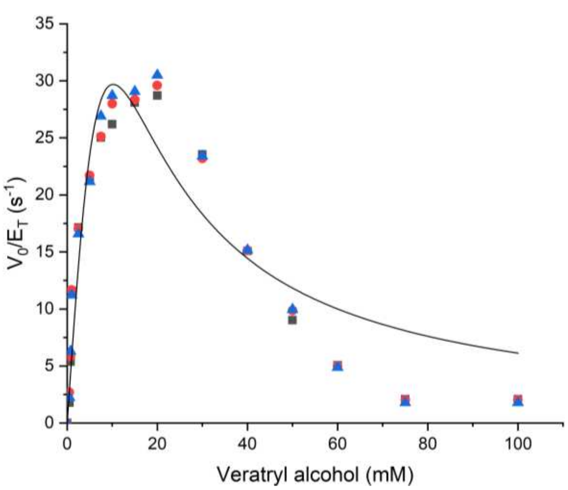
I.


V_0/E_T (s^{-1})
0 10 20 30 40
0 20 40 60 80 100
p-anisyl alcohol (mM)

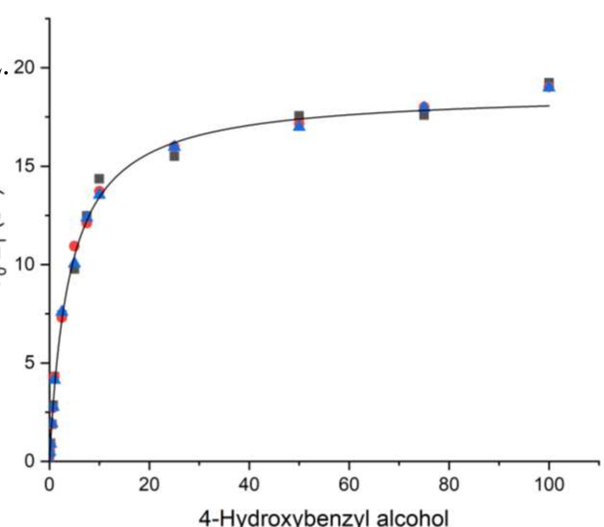
J.

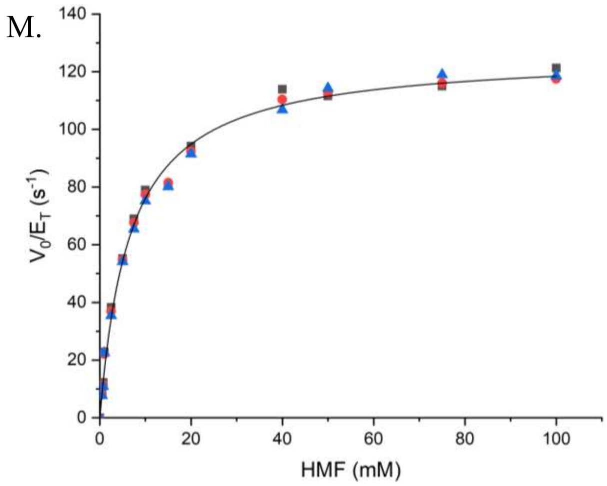

V_0/E_T (s^{-1})
0 20 40 60 80 100 120
0 20 40 60 80 100
m-anisyl alcohol (mM)

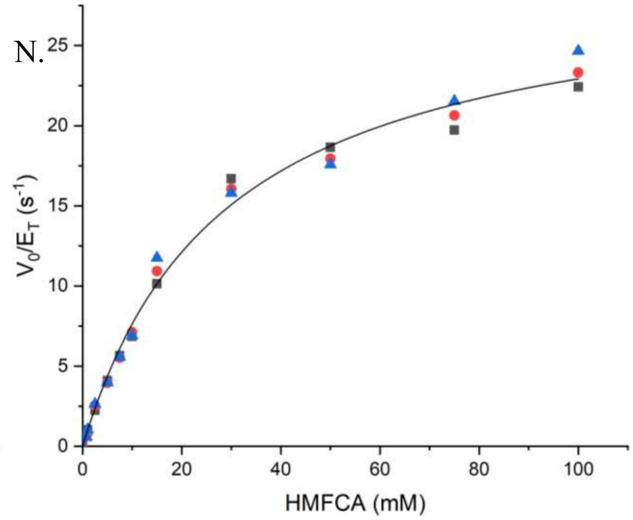
K.

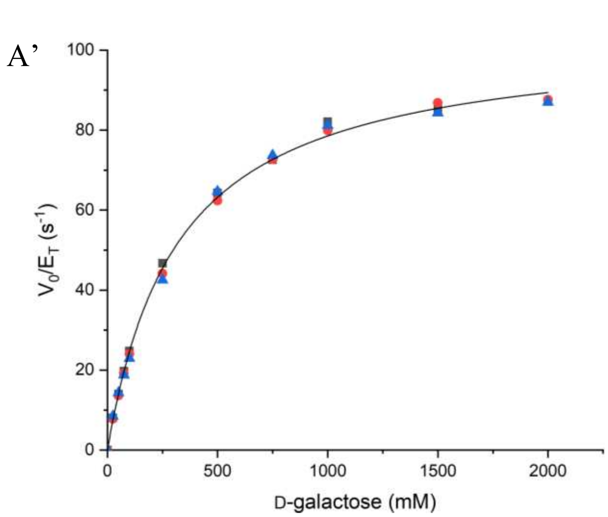

V_0/E_T (s^{-1})
0 5 10 15 20 25 30 35
0 20 40 60 80 100
Veratryl alcohol (mM)

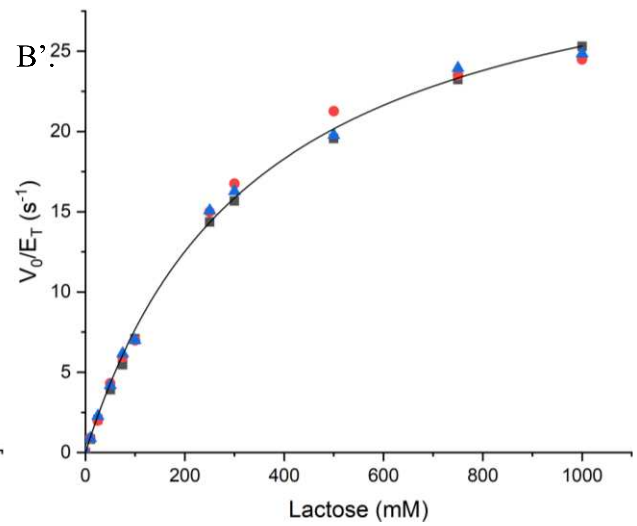
L.

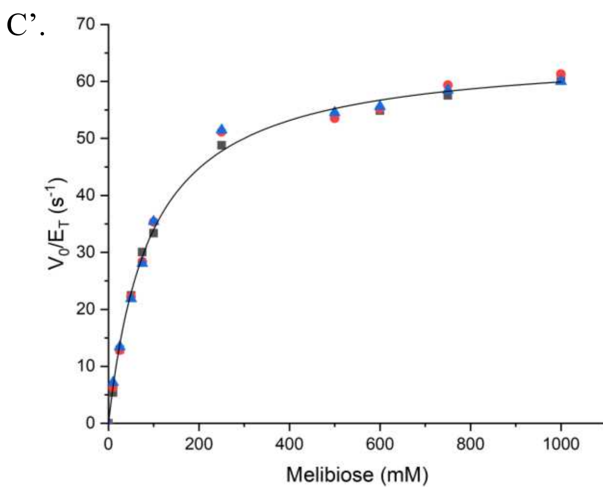

V_0/E_T (s^{-1})
0 5 10 15 20
0 20 40 60 80 100
4-Hydroxybenzyl alcohol

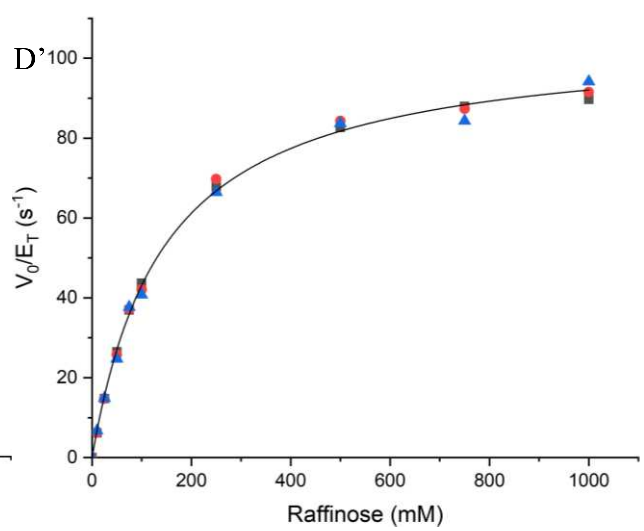

M.
V0/ET (s-1)
HMF (mM)

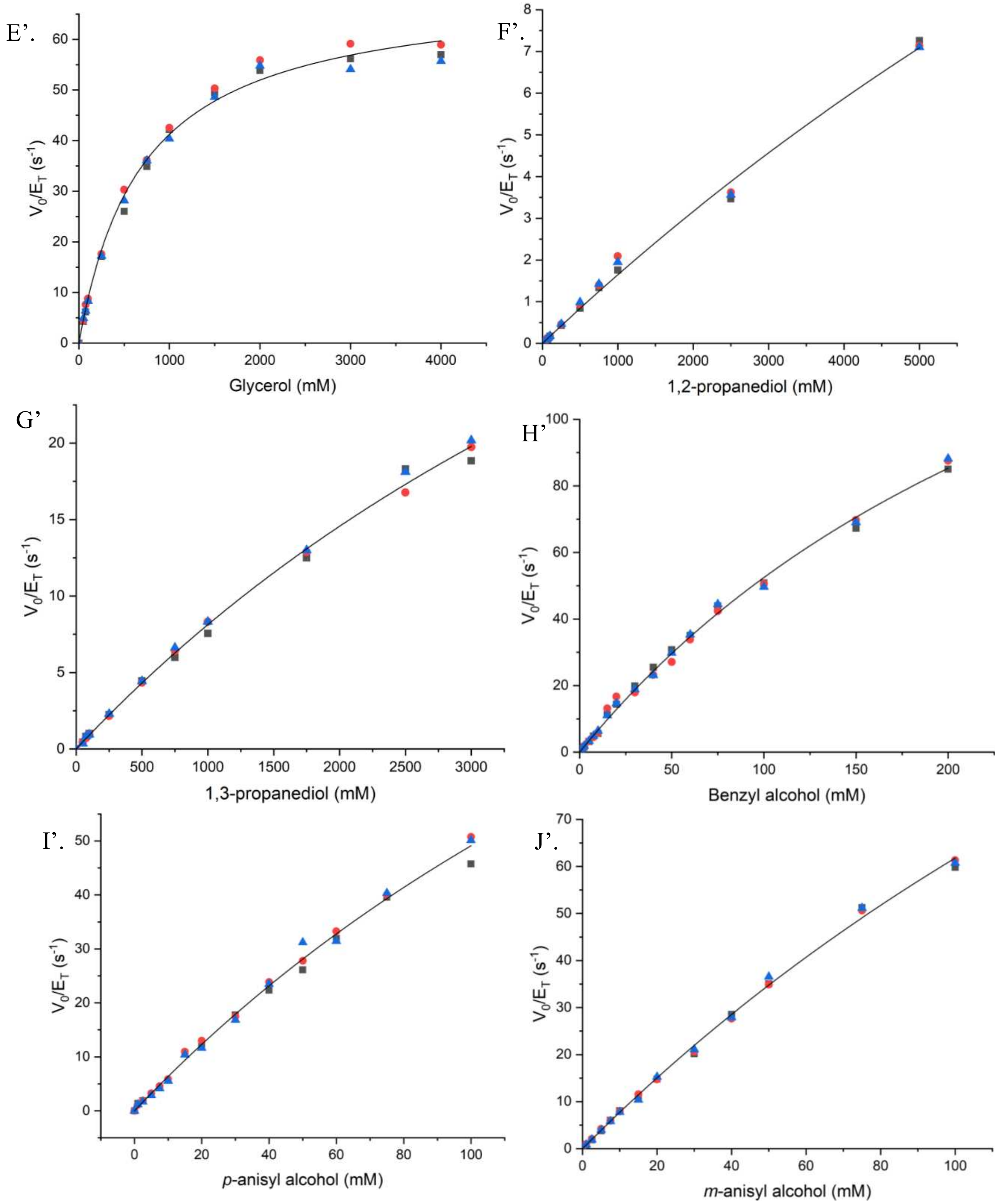

N.
V0/ET (s-1)
HMFCA (mM)

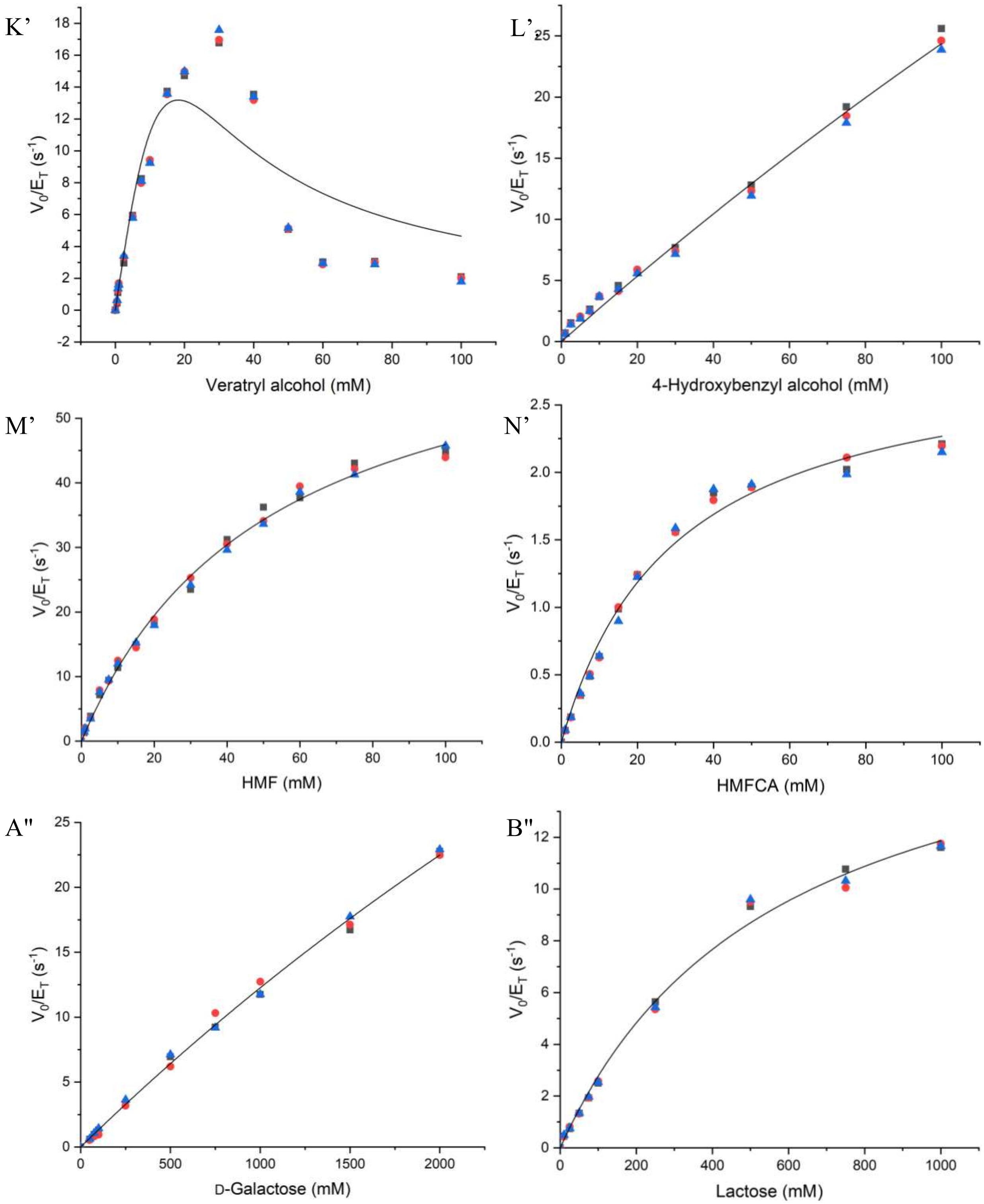

A'
V0/ET (s-1)
D-galactose (mM)

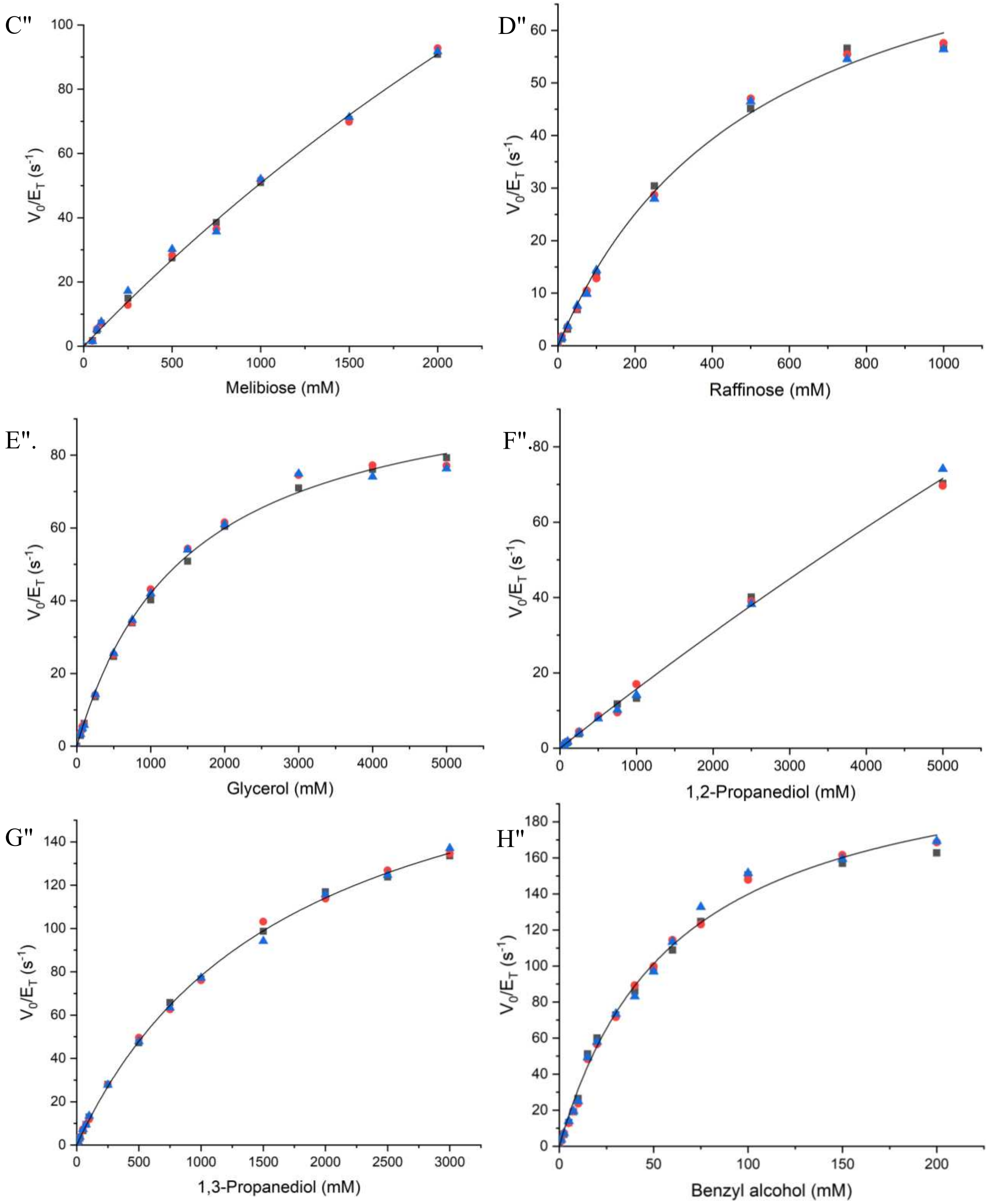

B'.
V0/ET (s-1)
Lactose (mM)

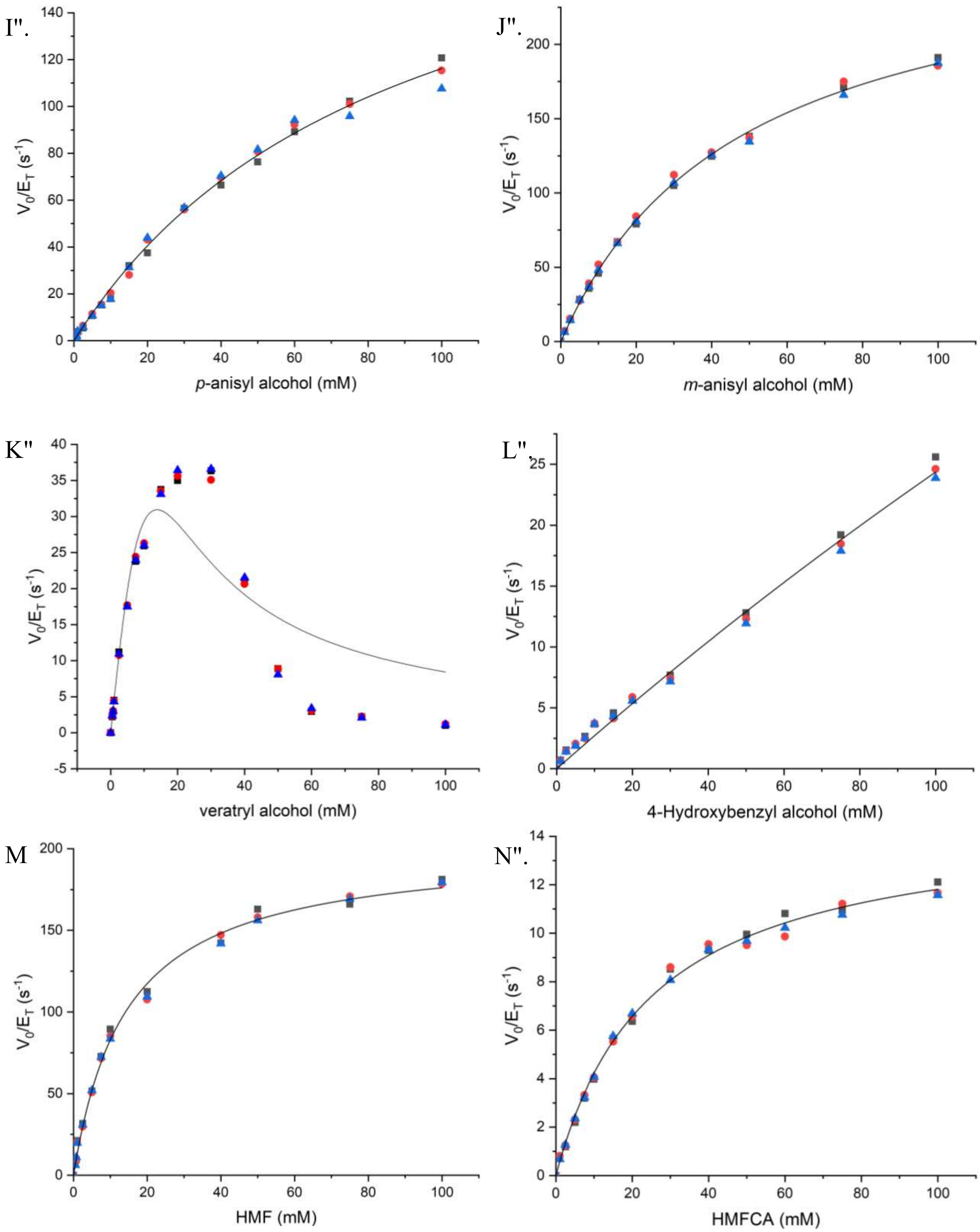

C'.
V0/ET (s-1)
Melibiose (mM)


D'
V0/ET (s-1)
Raffinose (mM)


E'.
F'.
G'
H'
I'.
J'.
V_0/E_T (s^{-1})
Glycerol (mM)
1,2-propanediol (mM)
1,3-propanediol (mM)
Benzyl alcohol (mM)
p-anisyl alcohol (mM)
m-anisyl alcohol (mM)

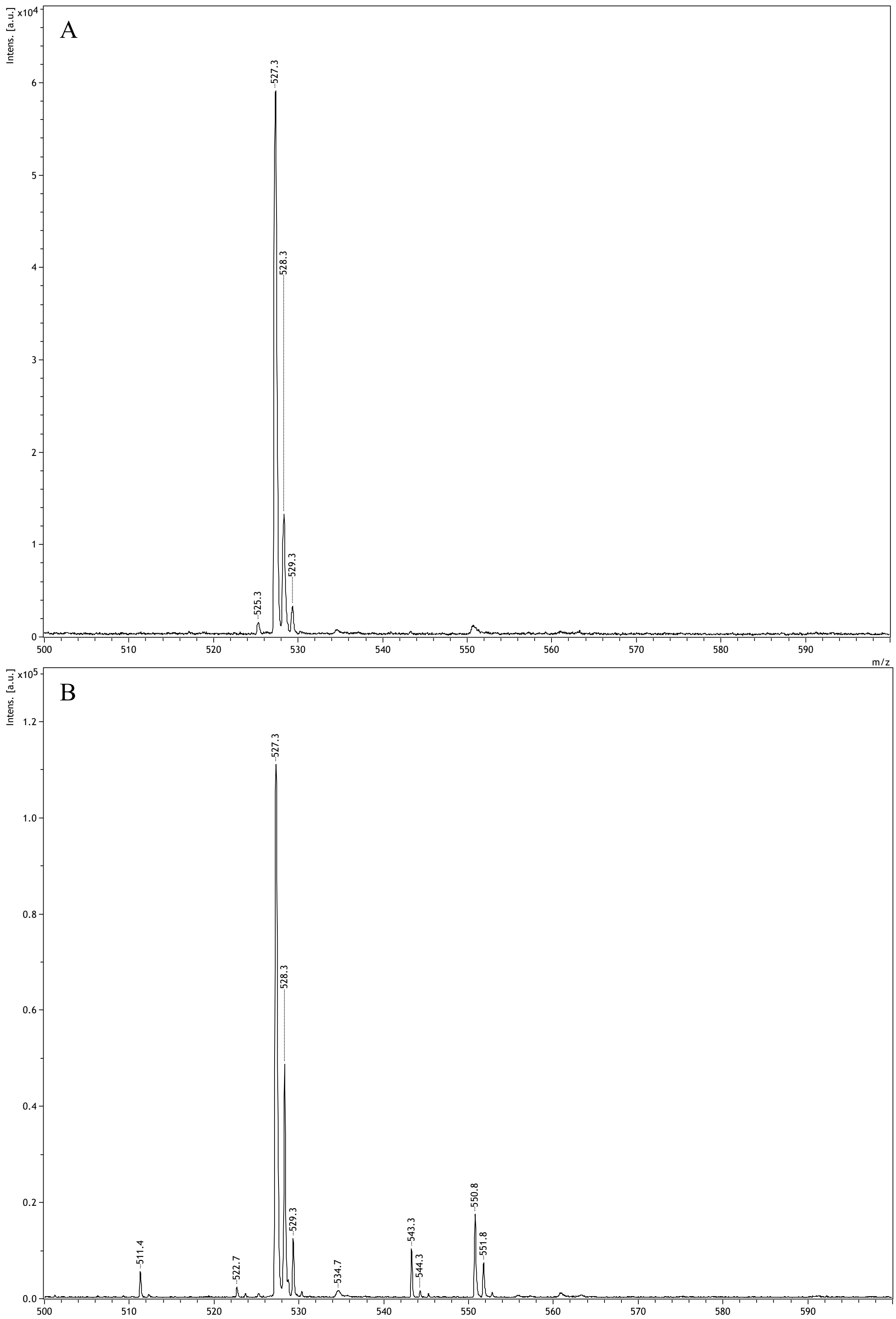

K’
L’
M’
N’
A"
B"
V0/ET (s-1)
Veratryl alcohol (mM)
4-Hydroxybenzyl alcohol (mM)
HMF (mM)
HMFCA (mM)
D-Galactose (mM)
Lactose (mM)

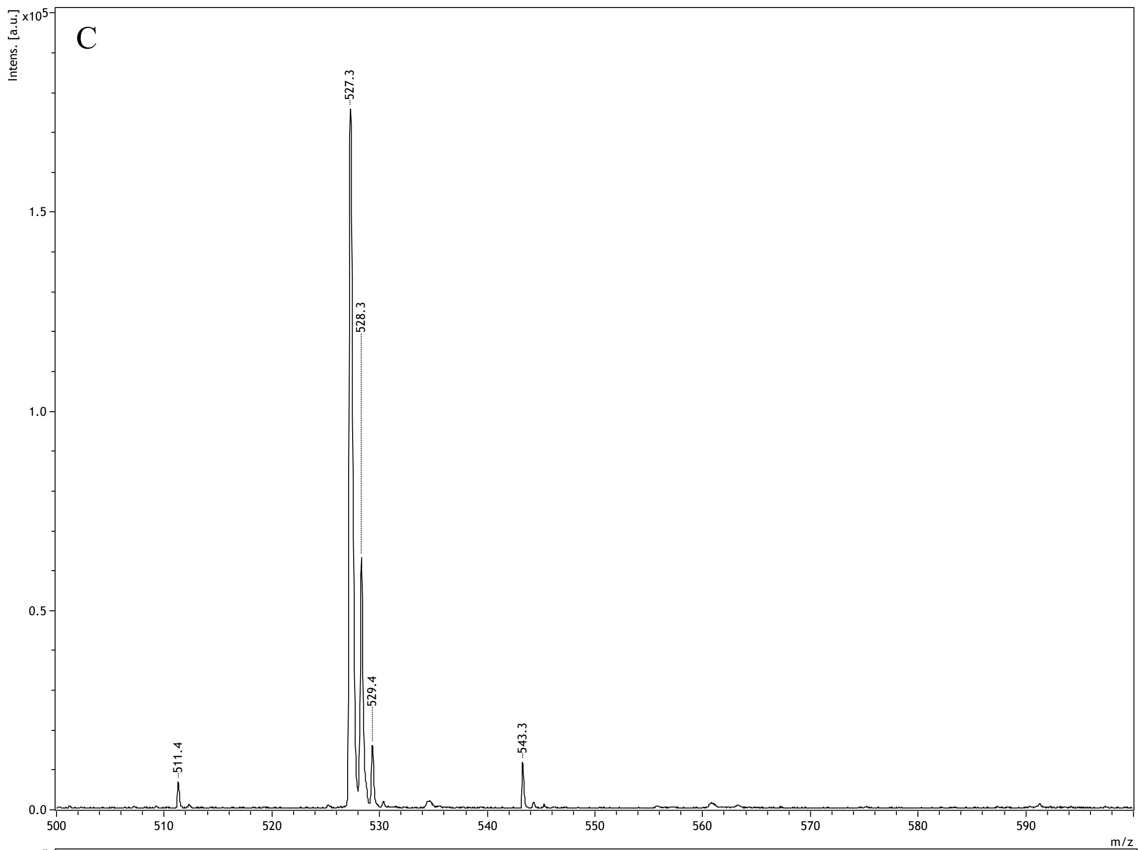

C"
D"
E".
F".
G"
H"
V_0/E_T (s^{-1})
Melibiose (mM)
Raffinose (mM)
Glycerol (mM)
1,2-Propanediol (mM)
1,3-Propanediol (mM)
Benzyl alcohol (mM)

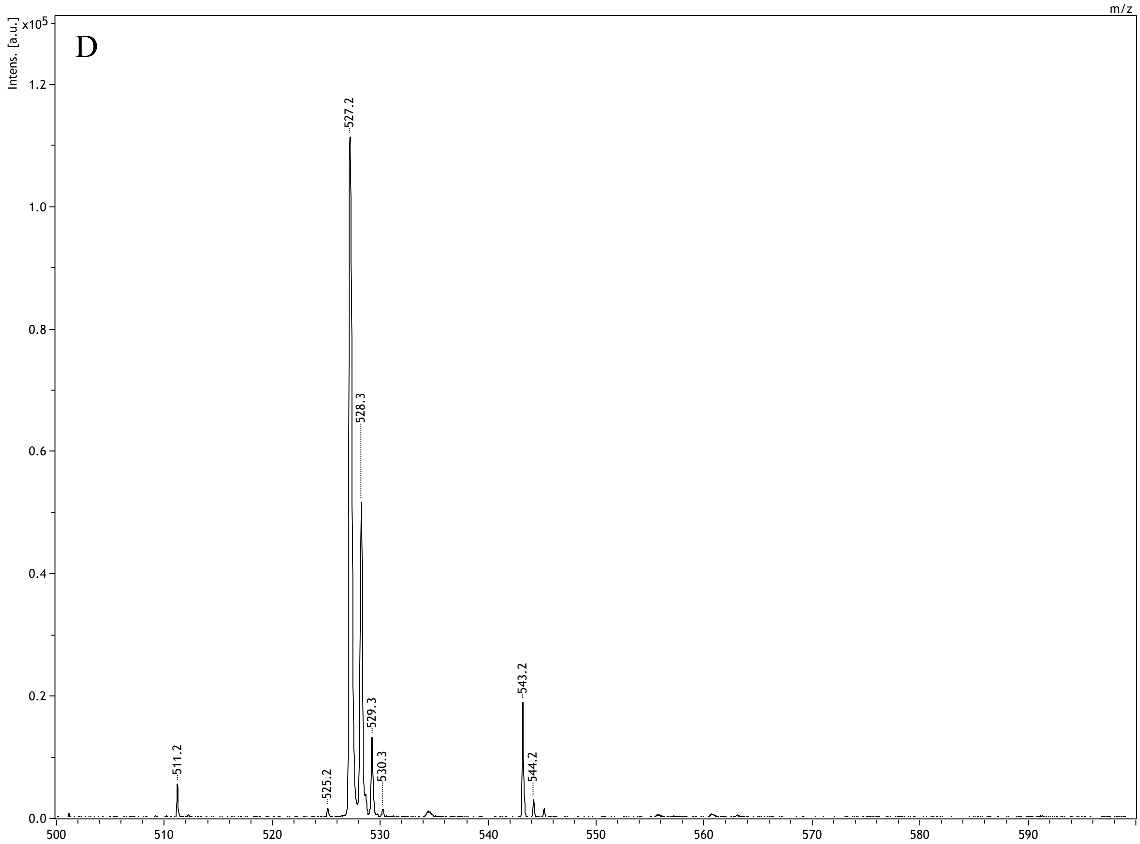


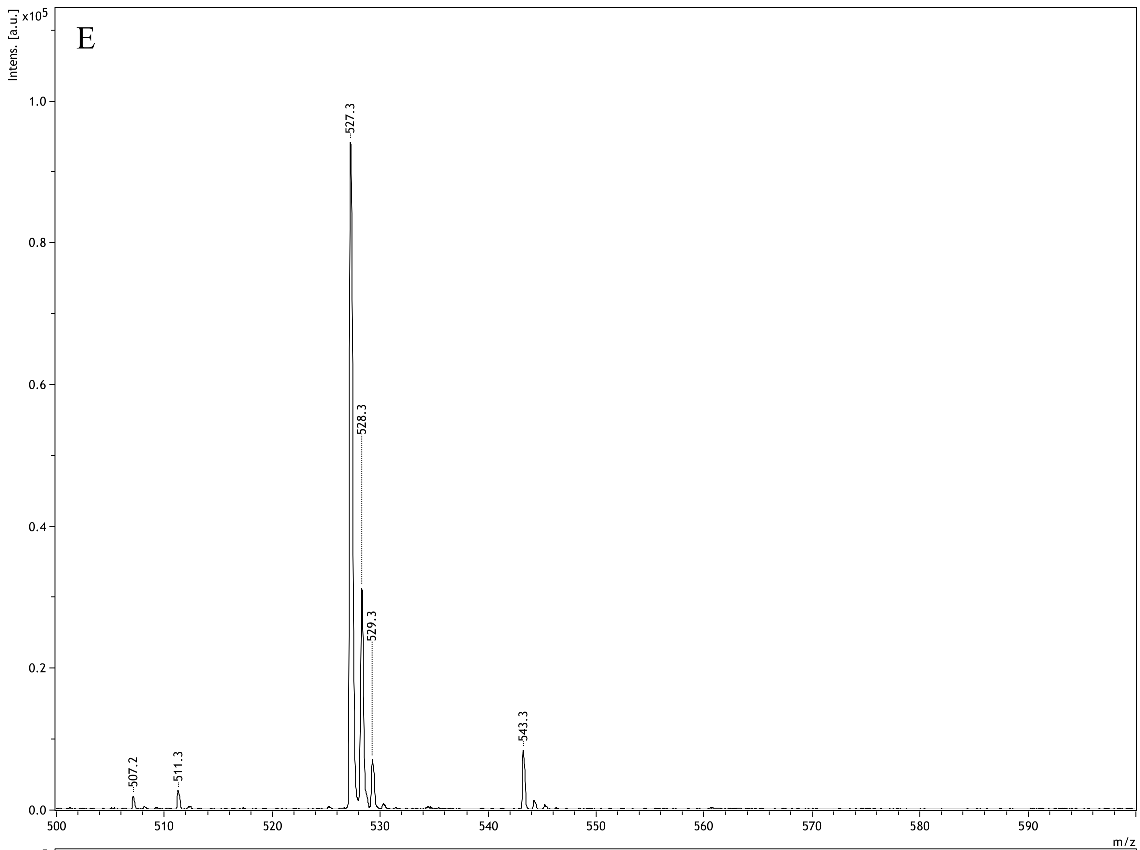
I".
p-anisyl alcohol (mM)
J".
m-anisyl alcohol (mM)
K"
veratryl alcohol (mM)
L".
4-Hydroxybenzyl alcohol (mM)
M
HMF (mM)
N".
HMFCA (mM)
V0/ET (s-1)

Figure S5. Initial-rate kinetics. Initial-rate values were measured in triplicate at each substrate concentration. Individual k_{cat} and K_m values were derived by non-linear fitting of the standard Michaelis-Menten or substrate-inhibition (veratryl alcohol) equations to the data using OriginLab 9.55. For substrates that did not display saturation kinetics, composite k_{cat}/K_m values were calculated from the slope of linear fits. Individual substrates are indicated in the x-axis labels of Panels A-N for *Cgr*AAO-WT; A'-N' for *Cgr*AAO-Y334W and A''-N'' for *Cgr*AAO-Y334F.

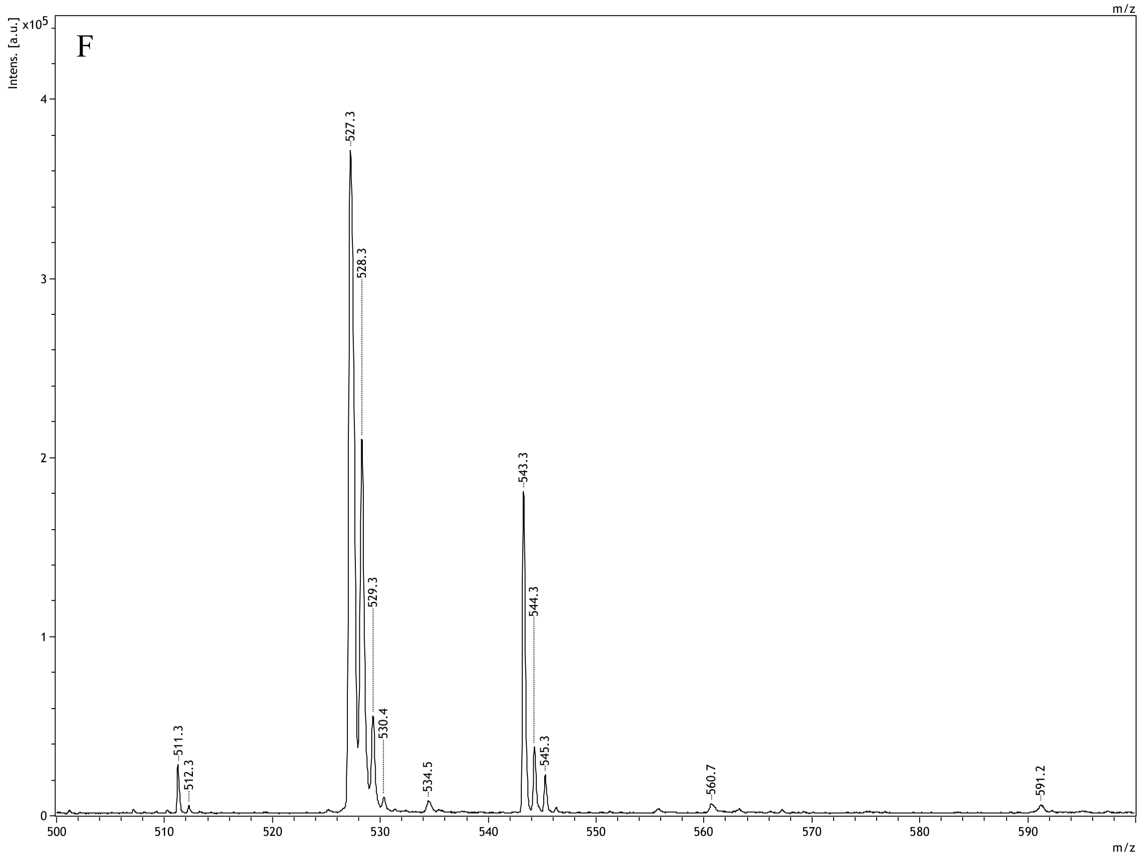

A
x10^4
Intens. [a.u.]
527.3
528.3
529.3
525.3
m/z
B
x10^5
Intens. [a.u.]
527.3
528.3
529.3
511.4
522.7
534.7
543.3
544.3
550.8
551.8
m/z


C
Intens. [a.u.]
x10^5
527.3
528.3
529.4
511.4
543.3
m/z

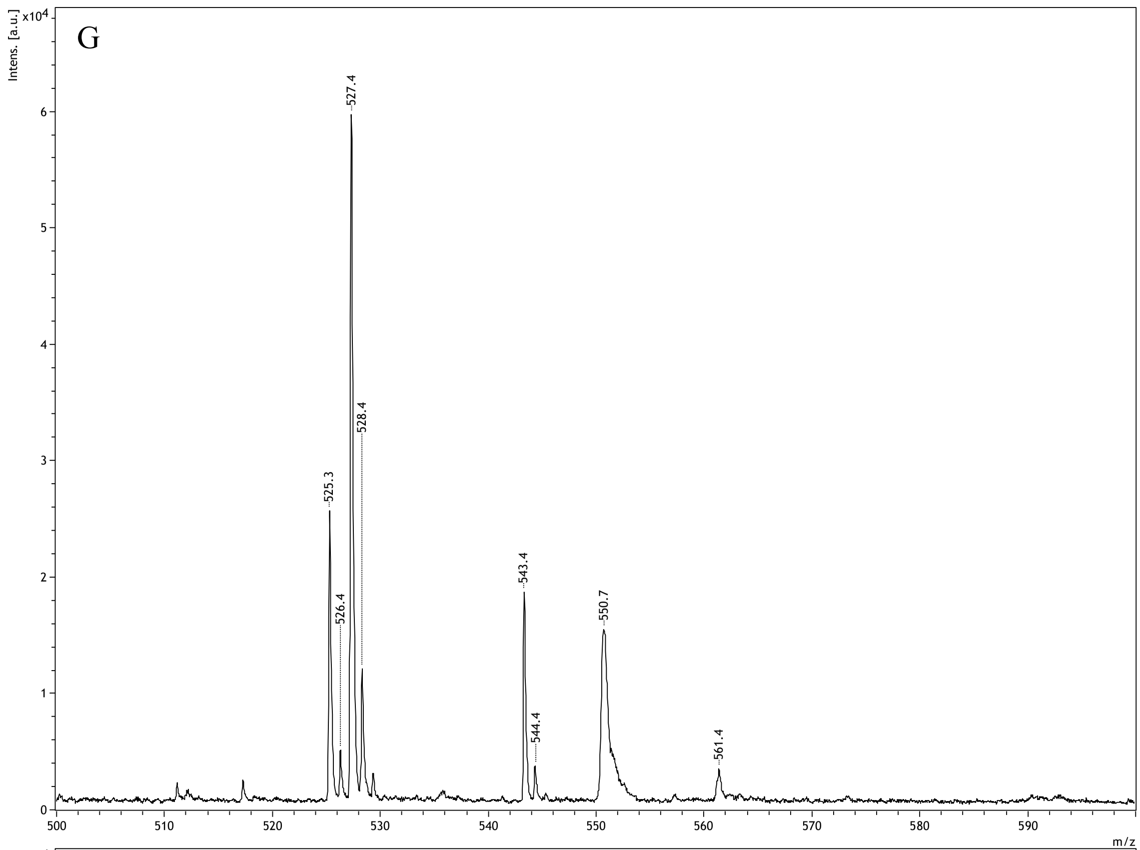

D
Intens. [a.u.]
x10^5
527.2
528.3
529.3
530.3
511.2
525.2
543.2
544.2
m/z

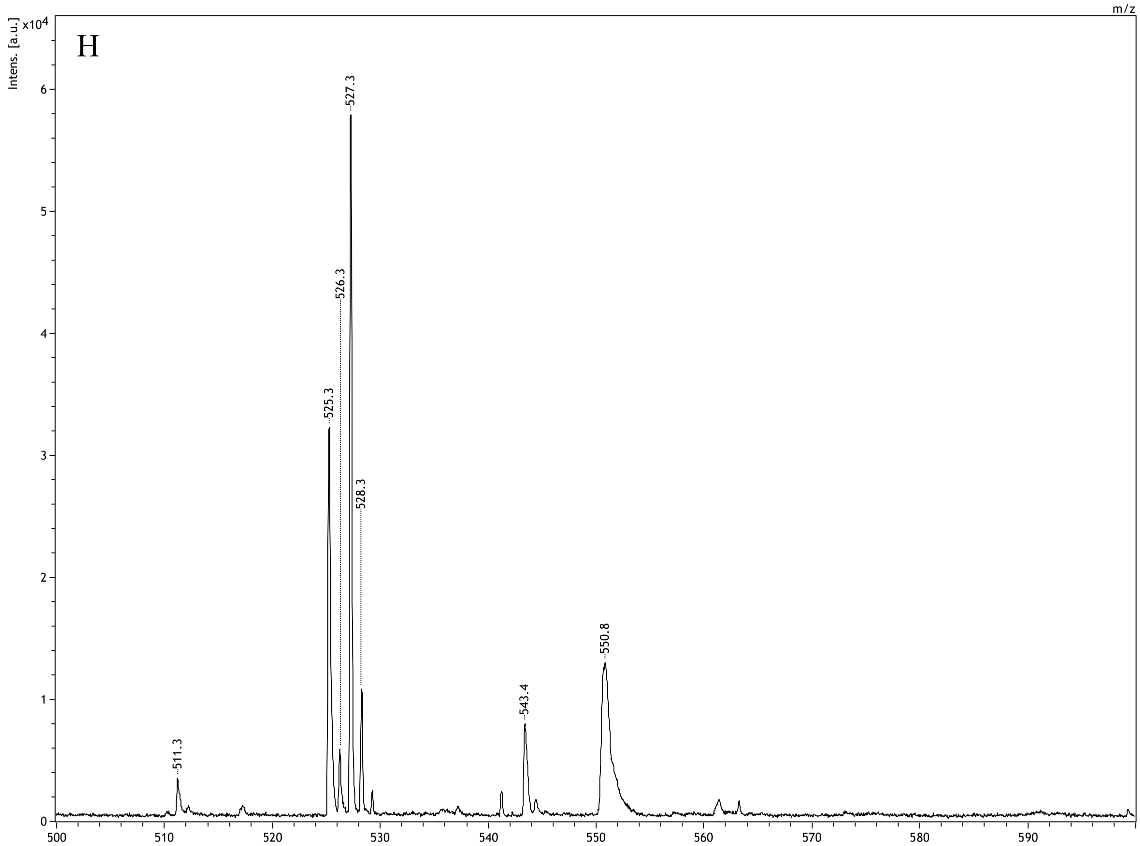


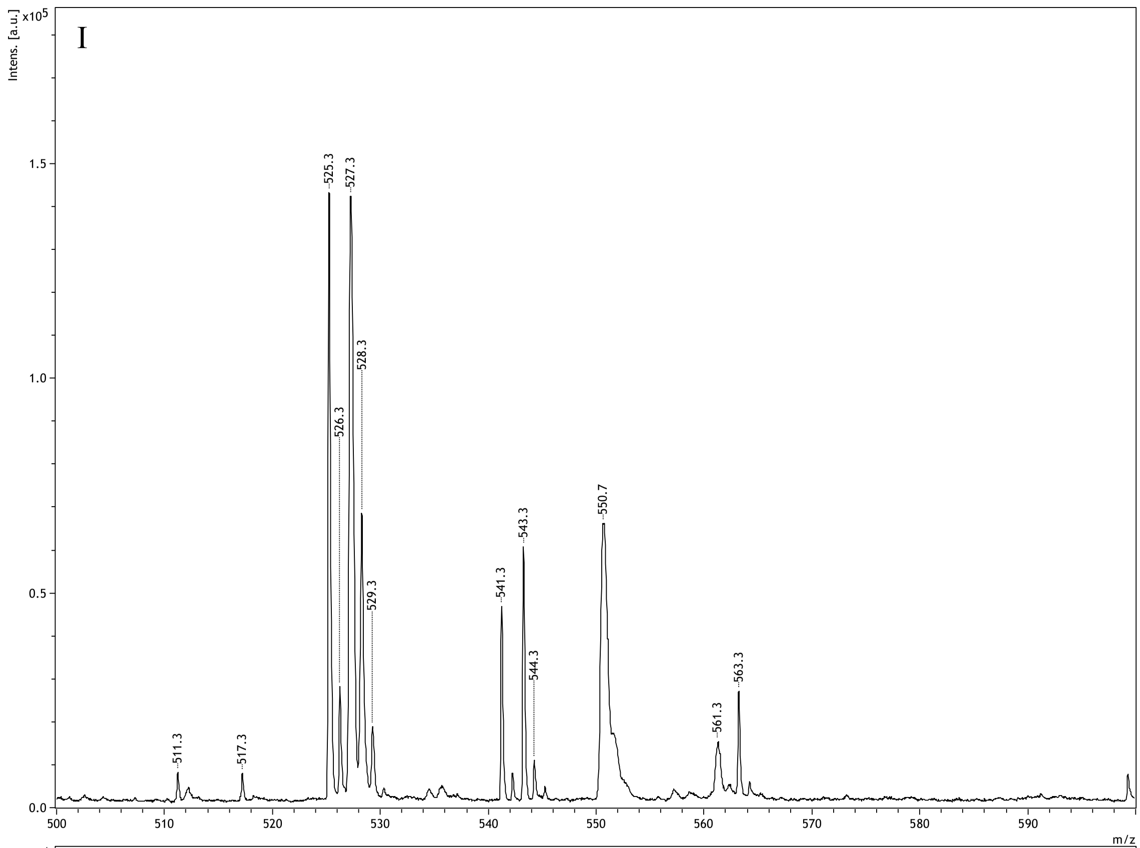
E
Intens. [a.u.]
x10^5
1.0
0.8
0.6
0.4
0.2
0.0
507.2
511.3
527.3
528.3
529.3
543.3
500
510
520
530
540
550
560
570
580
590
m/z



F
Intens. [a.u.]
x10^5
4
3
2
1
0
511.3
512.3
527.3
528.3
529.3
530.4
534.5
543.3
544.3
545.3
560.7
591.2
500
510
520
530
540
550
560
570
580
590
m/z


G
Intens. [a.u.]
x10^4
527.4
528.4
525.3
526.4
543.4
544.4
550.7
561.4
m/z


H
Intens. [a.u.]
x10^4
527.3
526.3
525.3
528.3
511.3
543.4
550.8
m/z


I
Intens. [a.u.]
x10^5
511.3
517.3
525.3
526.3
527.3
528.3
529.3
541.3
543.3
544.3
550.7
561.3
563.3
m/z

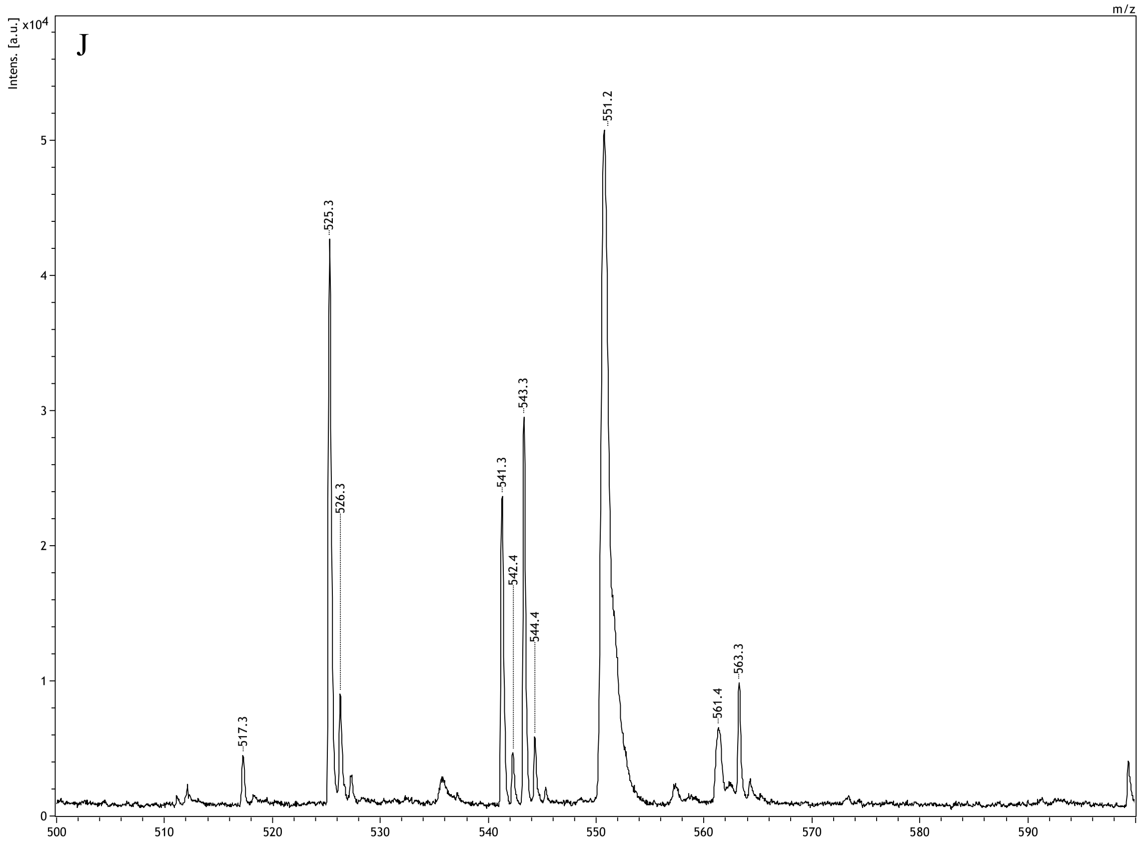

J
Intens. [a.u.]
x10^4
517.3
525.3
526.3
541.3
542.4
543.3
544.4
551.2
561.4
563.3
m/z

Figure S6. Time course analysis of raffinose oxidation by *Cgr*AAO by MALDI-TOF. (A-E) 10 mM raffinose incubated with 1 U of HRP/mg of substrate and 115 U of catalase/mg substrate at times 0 h (A), 2 h (B), 4 h (C), 8 h (D) and 16 h (E). (F-J) 10 mM raffinose incubated with 1 U of HRP/mg of substrate, 115 U of catalase/mg substrate and 200 µg of *Cgr*AAO at times 0 h (F), 2 h (G), 4 h (H), 8 h (I) and 16 h (J). m/z 527.3 = raffinose sodium adduct, m/z 525.3 = raffinose aldehyde product sodium adduct, m/z = 543.3 raffinose aldehyde product in hydrate form sodium adduct, m/z = 541.3 uronic acid derivative sodium adduct. The identity of the broad peak at m/z = 550.7 is unknown.

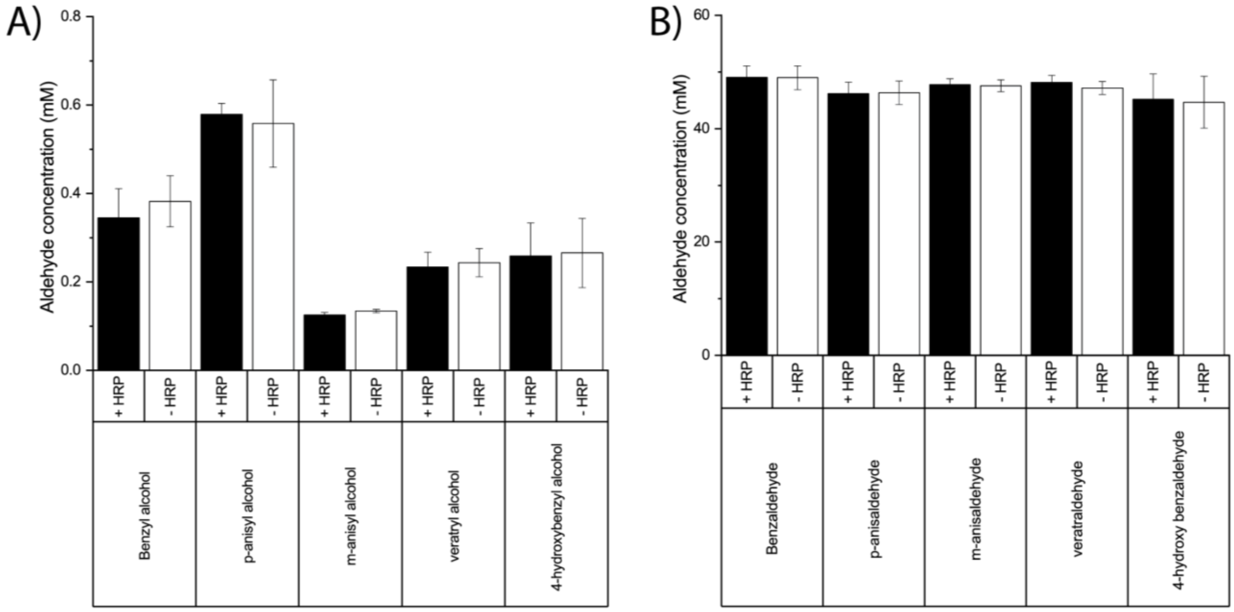


Figure S7. Aldehyde detection by Purpald. (A) 50 mM aryl alcohol incubated with 10 mM H_2O_2 in presence or absence of 2.3 μM HRP for 15 minutes. (B) 50 mM aryl aldehyde incubated with 10 mM H_2O_2 in presence or absence of 2.3 μM HRP for 15 minutes. Standard curves for each aromatic aldehyde made between 20 mM and 100 μM gave a linear response ($r^2 > 0.99$) with a limit of detection of 50 μM.

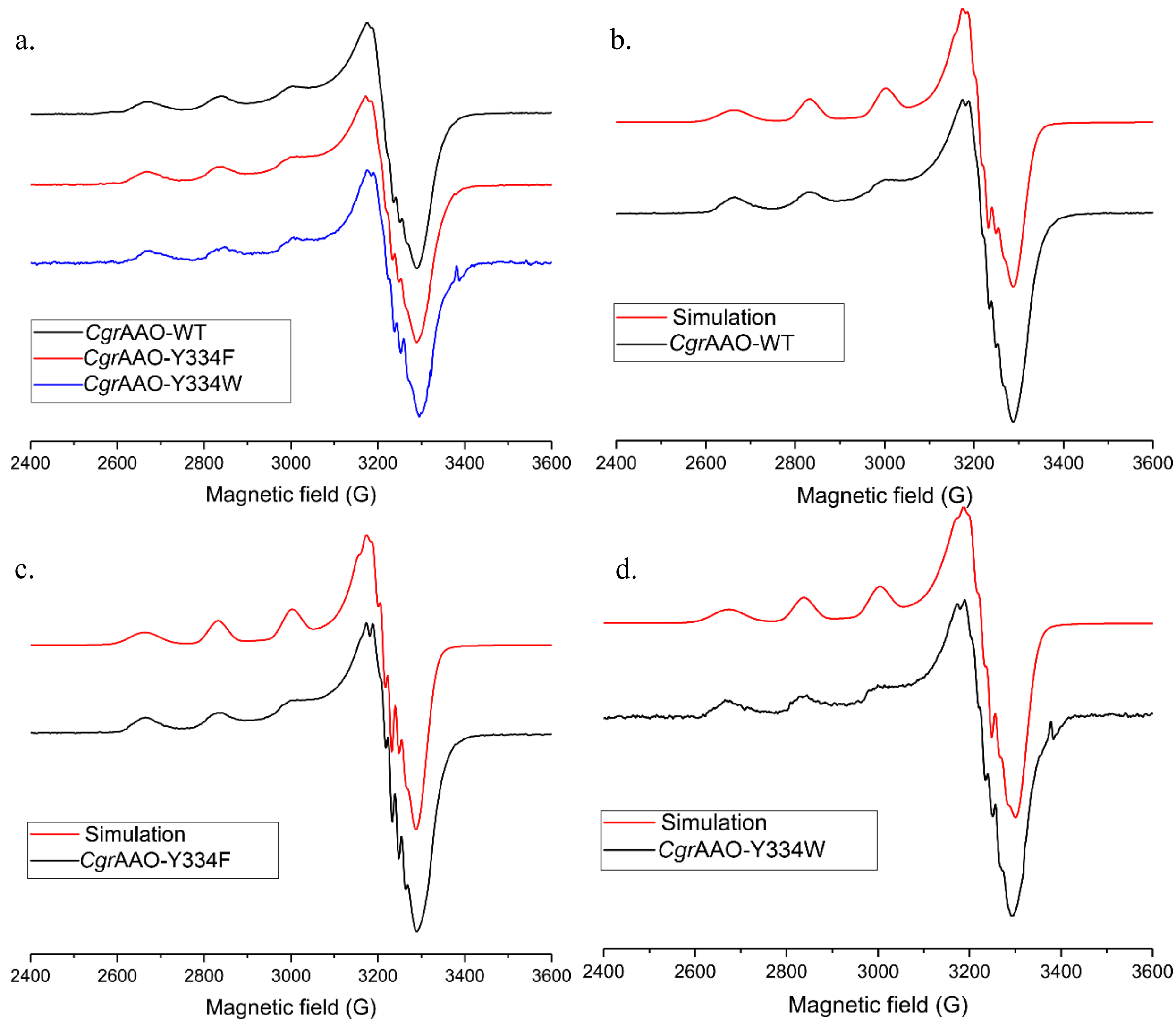


Figure S8: Continuous wave X band frozen solution spectra of *Cgr*AAO$_{(AA5_2)}$-WT, -Y334F and –Y334W collected in 100 mM Na phosphate buffer pH 7.0 without (a) and with 10% (*v/v*) glycerol (b, c, d). Simulations of the experimental data for *Cgr*AAO-WT (b), *Cgr*AAO-Y334F (c) and *Cgr*AAO-Y334W (**d**) are shown in red.

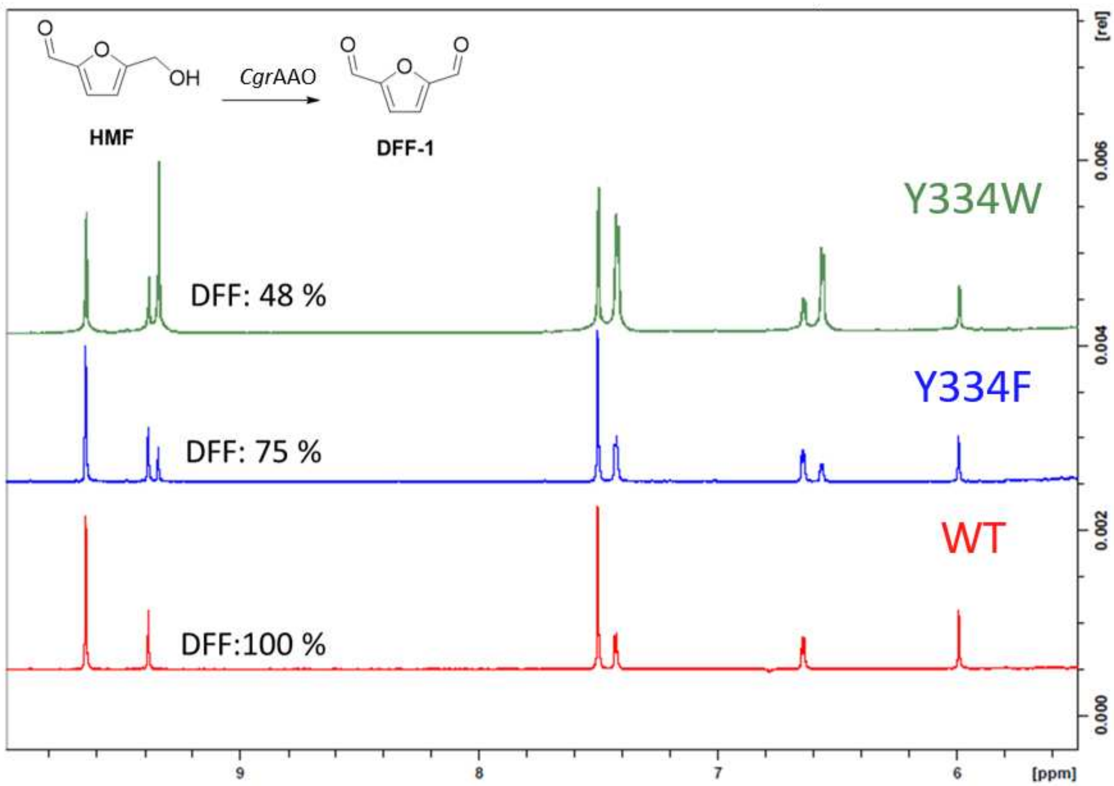


Figure S9. **^{1}H NMR spectra (400 MHz, 1:9 D_2O:phosphate buffer, 20 mM, pH 7) showing product profiles for the oxidation of 20 mM HMF by *Cgr*AAO wild type and variants, as indicated.**

Supporting References

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